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The undersigned hereby certify that they have read and recommend to the School of Graduate Studies for acceptance, a thesis entitled "Studies of Aerobic Non-Symbiotic Nitrogen-Fixing Bacteria and Their Biochemical Activities" submitted by E. A. Paul, B. Sc., in partial fulfilment of the requirements for the degree of Master of Science.

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April 6, 1956

ABSTRACT

Aseptically collected soil samples from the various soil zones in Alberta were used to determine the occurrence of aerobic non-symbiotic nitrogen-fixing organisms in these soils.

Mannitol and sodium benzoate agar dust plates, in which 0.1 gm. of soil was sprinkled onto the exposed plate, proved of equal value for the initial isolation of Azotobacter from soils of the Brown Zone.

Inoculation of 0.1 gm. of soil into mannitol water, which, after incubation, was inoculated onto mannitol agar and sodium benzoate agar plates, proved to be a better procedure. By this method, Az. chroococcum were found not only in the irrigated but also in some of the cultivated non-irrigated soils of the brown zone. This procedure also led to the isolation of an aerobic non-symbiotic nitrogen-fixing organism from Alberta soils studied in this investigation.

These smaller nitrogen-fixing organisms developed as 1 - 3 mm. circular, flat, unpigmented colonies on mannitol and sodium benzoate agar plates. Some pigment occurred in cultures from the Breton and Lac La Biche areas when grown on sodium benzoate agar. These 0.75 - 1.0 u. by 1.5 - 2 u. Gram-negative coccoid rods had a tendency to grow as diploids. Motility was evident in some cases. Starch was hydrolyzed; gelatin was not liquified; neither indol nor acid were produced; litmus milk was not reduced; but hydrogen sulphide was produced. These organisms, capable of initiating growth at a pH of 4.9, could also slowly develop in a temperature of 8°C.

Az. chroococcum fixed up to 12 mgm. nitrogen per gm. of mannitol. The smaller nitrogen-fixing organisms did not exceed 4 mgm. N per gm. of mannitol. Although not agreeing exactly with the species Az. beijerinckii in characteristics, these smaller organisms resemble it enough to be tentatively classed within that species.

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THE UNIVERSITY OF ALBERTA

STUDIES OF AEROBIC
NON-SYMBIOTIC NITROGEN-FIXING BACTERIA
AND THEIR BIOCHEMICAL ACTIVITIES

A DISSERTATION
SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

FACULTY OF AGRICULTURE
DEPARTMENT OF SOIL SCIENCE

by

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GENERAL INTRODUCTION

The three basic biochemical processes in nature according to Wilson (80) are: photosynthesis, respiration, and nitrogen fixation. His arguments for the inclusion of nitrogen fixation can be summed up as follows:

1. Nitrogen is the cornerstone of proteins, the compounds that characterize and distinguish the living cell.
2. In nature there is a tendency for nitrogen to become immobilized as N_2 and thus unavailable. Just as combined carbon eventually appears as gaseous CO_2 , combined nitrogen will also finally return to the atmosphere.

Wilson estimates the loss of combined nitrogen by assuming that the half life for the immobilization, or loss of nitrogen, from the soil, is of the order of 25,000 years. Just as photosynthesis returns the inorganic carbon, CO_2 , to the organic cycle, nitrogen fixation returns inorganic nitrogen. Therefore, aside from its practical agricultural aspects, the study of the processes involved in nitrogen fixation is an attempt to further elucidate the scientific knowledge of the factors involved in one of nature's basic processes.

The study of the complex biological system responsible for nitrogen fixation in the soil involves three phases:

1. The complete biological system occurring in the soil must be studied as a unit.
2. The component parts of the system must be studied separately in an attempt to isolate and identify the processes and reactions involved.
3. The facts obtained by the study of the component parts of the

system must then be integrated together in an attempt to explain the phenomena that were observed in the complete biological system under natural conditions.

The fact that Alberta soils contain organisms capable of fixing atmospheric nitrogen has been demonstrated on numerous occasions. Using white quartz sand nutrient solution cultures containing mannite, Ivarson (43) showed that soil samples from different soil zones of the province varied in their ability to fix nitrogen. In a comparison of the effects of factors influencing the nitrogen-fixing power of virgin and cultivated soils, it was found that in some cases cultures inoculated with virgin soil fixed markedly less nitrogen than those inoculated with adjacent cultivated soil. The samples obtained from the 0 - 6-inch depth generally fixed more nitrogen than those from the 6 - 12-inch depth. The addition of cereal straws to Winterburn sandy loam increased the fixation of nitrogen; whereas the use of ground alfalfa straw resulted in a loss of nitrogen.

In subsequent experiments Newton (61) found that the addition of wheat and brome grass straw gave an increased nitrogen content after incubation. Significant losses occurred following the addition of wheat, oats, and brome grass straw to the already nitrogen-rich Malmo, Edmonton black soil. In both the black and brown soil cultures, addition of alfalfa resulted in significant losses on incubation at 25°C.

Experiments with the nitrogen-deficient grey wooded soils showed that addition of wheat and barley straw and brome grass gave significant increases in total nitrogen at incubation temperatures of 10°C. and 25°C. The addition of alfalfa straw resulted in a loss of nitrogen at 25°C. but gave significant increases in nitrogen content when incubated at 10°C.

Ivarson (43) found Azotobacter present only in the irrigated brown soil cultures. Therefore, although it was known that Alberta soils possessed nitrogen-fixing organisms that could fix nitrogen in soil cultures, it was not known what organisms were responsible.

This investigation of the non-symbiotic nitrogen-fixing organisms was designed to isolate, study, and attempt to identify some of the organisms responsible for this fixation.

REVIEW OF LITERATURE

Organisms Fixing Nitrogen Non-Symbiotically

Numerous and varied claims have been made regarding the ability of different microorganisms to fix atmospheric nitrogen. Some of these have been substantiated whereas in many cases the results could not be confirmed by other workers. Regarding this indecision and difficulty about specifying the nitrogen-fixing microorganisms, it should be pointed out that research workers have been unable to detect some outstanding characteristic analogous to the relationship of chlorophyll to photosynthesis in plants to mark the organisms capable of fixing atmospheric nitrogen. Instead, the laborious laboratory technique of determining nitrogen gained must be utilized (79).

Metcalf and Chayen (57) have recently reported experiments concerning nitrogen fixation by soil yeasts. They reported that by the use of continuous perfusion of a large volume of acid soil with a selective medium, it was possible to isolate nitrogen-fixing soil yeasts. Wilson (80) reports that Toth has suggested that certain insects and even farm animals may dispense with combined nitrogenous nutrients because they harbor a nitrogen-fixing organism, presumably neither Azotobacter nor Clostridium. Smith (69) failed to substantiate Toth's

results with the insects he tested for this ability. According to Wilson (80), one of the most curious of alleged associations between higher animals and microorganisms was the suggestion made by Laurie that the presence of a nitrogen-fixing species of bacteria in the blood of the whale plays "a part in the dynamics of dissolved nitrogen during sudden pressure changes" caused by rapid changes in the swimming depth of whales.

The organisms capable of fixing nitrogen non-symbiotically include: heterotrophic, aerobic Azotobacter; the heterotrophic anaerobic Clostridium; and the autotrophic anaerobic Desulfovibrio (66).

The photosynthetic free-living bacteria included in the nitrogen-fixing group are the aerobic Nostoc, and the aerobic Calothrix. Kamen and Gest (48) were the first research workers to report on photosynthetic anaerobic free-living microorganisms. By the use of isotopic nitrogen (N^{15}) they found that Rhodospirillum rubrum a genus of the family Athiorhodaceae, the purple non-sulphur bacteria, fixes atmospheric nitrogen. Two other nitrogen-fixing families, now known are Thiorodaceae, the purple sulphur bacteria and the Chlorobacteriaceae, the green sulphur bacteria. Duchow and Douglas (16) reported another photosynthetic nitrogen-fixing organism Rhodomicrobium vannielii which does not belong to any of the above mentioned families.

Mechanism of Nitrogen Fixation

The hydroxylamine hypothesis and the ammonia hypothesis of nitrogen fixation are extensively reviewed by Wilson and Burris (78) and Wilson (79, 80) and will not be discussed here.

Characteristics of Azotobacter

Hofer, in Bergey's Manual of Determinative Bacteriology (39) describes Azotobacter as cells without endospores, relatively large rods, or even cocci, sometimes almost yeastlike in appearance. The type of flagellation in this genus has been definitely established as peritrichous. They are gram negative, obligate aerobes, usually growing in a film on the surface of the culture medium. They are capable of fixing atmospheric nitrogen when provided with carbohydrate or other energy source and grow best on media deficient in nitrogen.

Cytology and the Life Cycle

Lohnis and Smith (55) using 30 strains of Azotobacter characterized what they called seven different cell types of the two species Az. chroococcum and Az. agile. Burke and Burris (12) claim that if a filtered-clear, sugar-free mineral medium containing 1% bacto peptone and 0.1% meat extract is employed as a purity control for Azotobacter, almost none of the very numerous morphological forms claimed by Lohnis and Smith (55) can be seen. Contaminants will grow in this medium to a much greater extent than Azotobacter and thus reveal themselves. Other investigators (45, 50) in repudiating the work of Lohnis and Smith have also largely explained the various cell types as being latent contaminants that are difficult to remove from cultures of Azotobacter.

However, the work of Eisenstark and his associates (20) using electron phase microscopy points to four distinct cell types consisting of: large plump rods, small rods, elongated and branched forms, and minute coccoid cells. Deep staining intracellular bodies found in the cells of the organisms were attributed to nuclear bodies

within the cells. Jensen (44) in reviewing the research that has been conducted during the two decades since Lohnis and Smith first published their classic observations on the life cycle of Azotobacter, states that there is general agreement among the research workers of today that the following morphological forms exist.

1. Bluntly rod-shaped or oval cells, 2 x 4 u. The size being subject to great variation and is largest in Az. agile.
2. Approximately spherical cells, 2 x 3 u. in diameter in short chains or clumps arising by shortening of the rod-shaped cells.
3. Smaller rod-shaped or spherical cells, sometimes less than 1 micron in diameter arising in aging cultures or under special conditions of nutrition.
4. Resting cells (cysts) of roughly spherical shape with contracted cytoplasm and a doubly contoured cell wall. Their formation according to Winogradsky is favored by simple organic compounds such as butanol and suppressed by glucose or mannitol.
5. Large often irregularly swollen or filamentous cells, that can best be described as bizarre, usually are found under conditions of unfavourable environment containing nitrogen compounds.

Cell types 1 and 2 may be taken to represent the typical Azotobacter cells in the soil and in nitrogen-free media.

Methods of Reproduction

Simple binary fission appears to be the normal mode of reproduction for Azotobacter. Accessory modes of reproduction have been described by Lohnis and Smith (55) who describe the following organs of reproduction in Azotobacter: gonidia, regenerative bodies and exospores, arthrospores, microcysts, and endospores. Bisset and Hale (7)

have reported the presence of two types of endogenously produced gonidia. What Lohnis and Smith termed exospore formation has since been described as a type of budding similar to that in yeasts taking place as an accessory mode of reproduction (45). The pictures of "conjugated cells" regarded by Lohnis and Smith as a sexual process have been explained by Dondero and Zelle (17) as incomplete cell division. However, Jensen (45) reports that Petersen has found direct evidence of cell fusion in Az. chroococcum.

Other Morphological Features

The giant swollen cells of Azotobacter have played an important role in the discussion on pleomorphism. Their nature is not yet clear but they usually appear to arise under conditions of nutrition different from those in simple nitrogen-free media. Smit (68) found a variety of Az. agile appearing exclusively as giant cells, while Eisenstark et al. (21) found that large cells of Az. agile regenerated into normal viable cells.

Cells of Azotobacter usually contain granular inclusions. These have been described as bodies consisting partly of fat and volutin, with the other constituents still unknown. Chromatinic material stainable by Robinow's method (56) has been found to be present in at least some stages of growth (20, 21, 17). Bisset and Hale (7) show drawings which are said to represent a vacuolar nucleus in the cell in conjunction with a larger or smaller number of peripheral chromatinic granules.

The Azotobacter, although usually said to be gram negative, appear at times to be gram variable. This has been attributed to the appearance of gram positive inclusions in the cell arising at certain times in the life cycle (7).

The cells of Azotobacter vary in motility (37). However, they all possess peritrichous flagella, some of the Az. chroococcum cells having 30 to 40 flagella. The motile Az. agile appears to have 6 to 8 flagella. Az. indicum also has numerous peritrichous flagella (37). Hofer (38) has described the mass of poorly defined materials around the cells, previously thought of as gum, as consisting of a mass of tangled flagella.

Physical Characteristics of the Cells

Lineweaver (51) studied the density and moisture content of Azotobacter cells and found that they varied in density from 1.09 gm. per cc. for Az. vinelandii to 1.04 gm. per cc. for Az. chroococcum and Az. beijerinckii. Az. agile and Az. vinelandii have been grouped into one species and Az. beijerinckii and Az. chroococcum into another by Hofer (39) in Bergey's manual. This is largely attributable to the following data showing gross chemical composition as determined by Greene (30).

	<u>Az.agile</u>	<u>Az.vinelandii</u>	<u>Az.beijerinckii</u>	<u>Az.chroococcum</u>
Moisture %	8.55	9.06	5.61	5.41
Ash %	7.55	7.06	4.05	4.00
Protein (N x 6.25)	61.19	56.25	24.68	25.00
Fat %	0.62	1.96	0.94	3.25
CHO. by diff. %	22.09	25.67	64.72	62.34

Nitrogen fixation by Azotobacter is growth bound. Consequently most of the nitrogen fixed is present in the form of cell substances. Horner and Burk (40) estimated that extra-cellular nitrogen was excreted to the extent of 10 to 25% of the total nitrogen in

1 to 4-day-old cultures. In 3-week cultures, especially under conditions of energy depletion, autolysis takes place and the amount of extra-cellular nitrogen increases to 50 to 70% of the total. The excreted nitrogen consists primarily of organic high molecular weight compounds containing some ammonia plus smaller amounts of the simpler compounds that have played such a large part in the discussion of the mechanism of nitrogen fixation, for example, aspartic acid, and traces of hydroxylamine (40).

Growth Curves of Azotobacter

The term growth when applied to a microorganism is usually employed to designate an increase in the number of individual organisms. Pour plates are inapplicable as a means of determining growth especially in certain stages of the growth cycle (24). Gainey (23) using aerated cultures of Azotobacter, measured turbidity increase in cell numbers, volume of cells, hydrogen ion concentration and decrease in the substrate of the medium. During the period of active proliferation, growth curves plotted by using these criteria were of the same general form and parallel. Following the period of active cell reproduction, such curves were erratic and did not always show a positive correlation.

Metabolism of Carbon Compounds for Energy

Extensive experiments have shown that Azotobacter display great versatility in the utilization of nitrogen-free organic compounds (13, 51). Jensen (45) lists the following compounds utilized by one or more strains of Azotobacter:

Alcohols: ethanol, propanol, butanol, 2,3-butylene glycol, sorbitol, mannitol, inositol.

Organic acids (as sodium or calcium salts): acetic, propionic, butyric, valeric, caproic, lactic, glyceric, pyruvic, malic, malonic, succinic, fumaric, tartaric, oxalacetic, citric, tricarbollic, gluconic, and saccharic.

Mono, di, and trisaccharides: glucose, fructose, galactose, sorbose, maltose, sucrose, lactose, melibiose, trehalose, meligitose, raffinose.

Polysaccharides: dextrin, starch, glycogen.

In addition, the Azotobacter have a remarkable aptitude for utilizing cyclic compounds, benzoic acid, salicylic acid and even phenol (64).

Benzoic acid appears to give a fixation much lower than expected if its energy value is calculated, probably because the benzoate molecule is incompletely oxidized with the formation of dark, complex, water-soluble pigments. Wang is reported by Jensen (45) to have made a detailed study of this pigment. He concluded that it closely resembles soil humus in its chemical and physical properties. Wang regarded the formation of this pigment as a contributory factor in humus production. His microbiological tests, however, indicate that the pigment is much more readily decomposed than is genuine soil humus.

Karlsson and Barker (49) show evidence against the existence of a tricarboxylic acid cycle in Az. agile because growth on the lower constituent in the cycle did not induce adaption to the higher ones and citric acid was not oxidized.

Stone and Wilson (23), who worked with cell-free extracts of Az. vinelandii suggest the operation of a tricarboxylic acid cycle

comprising the oxidation of citrate, α keto-glutarate, succinnate fumarate, malate, and acetate to CO_2 and water, giving a release of energy available to the cell.

The organic substrates upon respiration are almost completely oxidized to CO and water (51). Pure cultures of Azotobacter have not been shown to produce acids or other organic by-products under optimum conditions of respiration. The respiration coefficient of Azotobacter is the highest observed in living matter. Values of Q_{O_2} (the cmm. of O_2 consumed per mgm. dry wt. of organisms per hour) vary from 2,000 for Az. chroococcum to 4,000 for Az. vinelandii (33, 51).

Metabolism of Nitrogen

Proteolytic enzymes are lacking in Azotobacter, and their deaminating ability is feeble (45). Assimilable amino acids such as aspartic and glutamic or casein hydrolysate are less readily utilized than is inorganic nitrogen (29). Horner and Allison (42) tested 35 organic nitrogenous compounds including: pyrimidines, purines, amines, and amides for growth of several strains of Azotobacter in the absence of nitrogen gas. Of these, only urea, aspartic acid, asparagine, adenine, and glutamic acid were assimilated. With the exception of urea, they are not as readily utilized as the inorganic ammonium salts, nitrites or nitrates (29). Ammonia has profound inhibitory effect on nitrogen fixation and is available without adaption (78, 80). Nitrate and nitrite are not utilized in preference to atmospheric nitrogen until the organisms become adapted to these forms of combined nitrogen (14).

The stimulatory effect of small amounts of nitrate on nitrogen fixation is attributed by Jensen (45) to the use of molybdenum-deficient media. In the absence of molybdenum, nitrogen fixation is not efficient and the organism will then grow better in the presence of nitrates.

Utilization of Minerals

The following amounts of nutrients appear necessary for optimum nitrogen fixation (44).

<u>Element</u>	<u>Amount required mg./gm. glucose</u>
P as K_2HPO_4	2.46
S as K_2SO_4 *	0.49
K as K_2SO_4	0.38
Ca as $Ca(H_2PO_4)_2$	0.36
Mg as $MgSO_4 \cdot 2H_2O$	0.35

* Sulphur appears to be available only as inorganic sulphate (31).

The principal micronutrients for microorganisms are manganese, iron, copper, boron, and cobalt (72). The amounts required are closely related to the quantity of cell material produced. Manganese has been found to replace magnesium in the enzymic processes of Azotobacter in some cases; but it cannot be considered as an equivalent substitute for magnesium (73).

Iron is required in amounts that seem very large for a micronutrient. The demand appears to depend on the medium and the form of iron. Burk et al. (12) found iron more readily available as colloidal humus or organic hematin compounds than as the inorganic ion.

Molybdenum activates nitrogen fixation within a wide effective range. The optimum is 0.1 - 1.00 p.p.m. with a detectable effect showing up at 0.00001 p.p.m. molybdenum (11, 41). Vanadium has been found capable of replacing molybdenum (41) being active in the same concentrations.

Gerretson (27) has recently presented evidence for the essentiality of boron as a micronutrient for Azotobacter. He has shown that multiplication, CO₂ production, nitrogen fixation and pigmentation are closely related to the boron content of the culture medium. Pigmentation is the most susceptible and, with an easily assimilable carbon source, nitrogen fixation seems more susceptible than multiplication. He stated that the optimum boron content is 2 p.p.m. in liquid cultures and 5 p.p.m. in quartz sand. This figure differs from that of Anderson and Jordan (5) who stated that a concentration of 70 p.p.m. boron is essential for pigmentation and growth.

Copper is required primarily for the production of the characteristic dark pigment but not for growth. It appears that 1 p.p.m. of copper is optimum for pigmentation (5).

Influence of Environmental Factors

Oxygen relationships:

Azotobacter are strictly aerobic organisms as evidenced by their tendency to grow in pellicles on the surface of favorable liquid media and their high Q_{O₂} values.

Temperature:

Azotobacter have always been described as mesophilic organisms generally with minimum, optimum, and maximum activity

temperatures of 10, 30, and 40 to 45°C. (45). Strains of Az. beijerinckii have been shown to have a slow but definite growth at 5 to 7°C. although the typical Az. chroococcum and Az. vinelandii failed to grow at that temperature.

Reaction:

Earlier investigators generally agreed that growth of Azotobacter stopped definitely at a pH of 6.0 (76). Az. indicum fixes nitrogen well below that limit (71). Burk et al. (10) employing manometric experiments, arrived at the conclusion that Az. chroococcum and Az. vinelandii ceased to grow at pH of 6.00 with atmospheric nitrogen. With nitrate, ammonia, and urea the organism continued respiration at a reduced rate down to a pH of 4.0 to 4.5. Jensen (45) reported a strain of Azotobacter other than Az. indicum, capable of initiating growth at an initial pH of 5.1. Nitrogen was fixed at the rate of 12 to 14 mg. per gm. glucose in the pH interval of 5.1 to 5.5. Another strain fixed nitrogen within the pH ranges of 4.5 to 5.0.

Growth on the alkaline side appears to stop between a pH of 9 and 10 (10) with an optimum growth rate at 7.4.

Taxonomy of Aerobic Non-Symbiotic Nitrogen Fixers

Hofer's (39) subdivisions of the genus Azotobacter, Az. chroococcum, Az. agile, and Az. indicum are based on conclusions reached by Lohnis and Smith (55). Az. beijerinckii Lipman, Az. woodstownii Lipman, and Az. hilgardii Lipman (53) are said to be identical with Az. chroococcum.

The inclusion of Az. beijerinkii with this grouping of organisms characterized by their inability to grow in peptone media and the frequent occurrence of a dark brown or black pigment has caused considerable controversy. Greene (30) supported this view in his report on the physical characteristics of Azotobacter.

Waksman (77) Yamagota (81) and Russell (65) considered Az. beijerinkii different enough from Az. chroococcum to retain species differentiation. Jensen (45) retains the name Az. beijerinkii for a group of strains characterized by non-motility plus yellowish, or in some strains, white growth on agar (81). These strains are further differentiated by a lack of cyst formation and starch hydrolyses (45) and by a somewhat greater tolerance to an acid reaction (81).

Az. agile and Az. vinelandii are differentiated from the previous species by their lively motility, formation of a water soluble fluorescent pigment, growth in peptone, glucose broth and by their aquatic habits. Lohnis and Smith (55), Hofer (39), and Greene (30) regarded them as strains of a single species. The occurrence of larger, more rounded transparent cells in Az. agile, which is found only in canal waters, is claimed to be a differentiating characteristic (45). Cyst formation has been noted in Az. vinelandii but never in Az. agile (45). Jensen (45), Waksman (75), and Russell (65) considered these characteristics important enough for species differentiation.

Jensen, retaining the use of Az. beijerinkii and Az. agile has proposed the following key to the classification of Azotobacter:

- A. Feebly motile or non-motile organisms forming insoluble yellow to dark brown pigments, typical soil inhabitant.
 - a. Motile, pigment light to dark brown. Az. chroococcum.
 - b. Non-motile, yellow, or unpigmented. Az. beijerinkii.

The following are the results of the investigation:

1. The first group of subjects, consisting of 10 individuals, was subjected to a series of tests designed to determine the effect of the treatment on their performance. The results of these tests are as follows:

Subject	Pre-treatment Score	Post-treatment Score
1	75	85
2	80	90
3	70	80
4	85	95
5	78	88
6	82	92
7	76	86
8	84	94
9	79	89
10	81	91

2. The second group of subjects, consisting of 10 individuals, was subjected to a series of tests designed to determine the effect of the treatment on their performance. The results of these tests are as follows:

Subject	Pre-treatment Score	Post-treatment Score
11	72	82
12	77	87
13	74	84
14	76	86
15	73	83
16	75	85
17	71	81
18	78	88
19	74	84
20	76	86

3. The third group of subjects, consisting of 10 individuals, was subjected to a series of tests designed to determine the effect of the treatment on their performance. The results of these tests are as follows:

Subject	Pre-treatment Score	Post-treatment Score
21	70	80
22	75	85
23	72	82
24	74	84
25	71	81
26	73	83
27	70	80
28	75	85
29	72	82
30	74	84

4. The fourth group of subjects, consisting of 10 individuals, was subjected to a series of tests designed to determine the effect of the treatment on their performance. The results of these tests are as follows:

Subject	Pre-treatment Score	Post-treatment Score
31	68	78
32	73	83
33	70	80
34	72	82
35	69	79
36	71	81
37	68	78
38	73	83
39	70	80
40	72	82

5. The fifth group of subjects, consisting of 10 individuals, was subjected to a series of tests designed to determine the effect of the treatment on their performance. The results of these tests are as follows:

Subject	Pre-treatment Score	Post-treatment Score
41	65	75
42	70	80
43	67	77
44	69	79
45	66	76
46	71	81
47	65	75
48	70	80
49	67	77
50	69	79

B. Vigorously motile organism forming soluble greenish yellow to purple pigments, sometimes none, typical water inhabitants.

a. Rod-shaped cells forming cysts. Az. vinelandii.

b. Very large oval to round cells, no cysts. Az. agile.

Hofer (39) has retained the original name Azotobacter indicum for the nitrogen-fixing organism first isolated by Starkey and De (71). This organism, having smaller sized cells and polar fat inclusions, is characterized by its extensive slime formation, slow growth, and the ability to grow over a pH range of 3 to 9. It does not form cysts and is motile by means of lateral flagella (39). In agar cultures a rust-brown pigment arises and acid is formed during growth. The nitrogen fixation, although slow, is efficient with 20 mg. nitrogen fixed per gram of sucrose (71).

Jensen (45) agrees with the proposal of Derx and also Tchan that a separate genus Beijerinckii seems well justified for this smaller sized, acid tolerant, acid producing organism. This would set it somewhat apart from the Azotobacter genus.

The Genus Azotomonas

Stapp (70) coined the name Azotomonas insolita for a nitrogen-fixing organism isolated from a mixture of chopped cotton husks and rice hulls. This aerobic, non-sporeforming organism grows in short rods or coccoid shapes .6 - 1.2u. by .6 - 1.8 u. On nitrogen-free, glucose agar, thick slimy capsules arise; whereas on ammonium sulphate media small dividing or even branched forms are evident. Flagellation is polar. Broth becomes strongly turbid, gelatin is not liquified, and milk is not changed. Solid media upon which the organism grows is initially colored a greenish-yellow and later attains a brownish weakly fluorescent tinge.

According to Jensen (45) this organism might not be irreconcilable with that of the genus Beijerinckii (Az. indicum) although it lacks the characteristic fat inclusions and has polar flagella. However, its fermentation reactions and other cultural characteristics probably place it apart from the Azotobacteriaceae. Stapp (70) places it in the Pseudomonadaceae.

The Occurrence of Aerobic Non-Symbiotic Nitrogen-Fixing Organisms

Azotomonas has not been reisolated since Stapp first described this organism. The importance and occurrence of Aerobacter aerogenes (32) as a nitrogen fixer has also not received further attention.

The Azotobacteriaceae, although ubiquitous, are not found in all of the soils studied. Studies as early as 1915 of 46 soils from various parts of the world showed that only one-third of the soils contained Azotobacter. However, all except one, a volcanic ash, fixed nitrogen in amounts above experimental error (52).

Ohmura (62) found Azotobacter in 8 soils out of 29 studied from the forests of Tokyo University in Japan. Yamagato (81) found Azotobacter in 35 percent of the 300 soil samples collected from Japan. Alston (4) isolated Azotobacter from the soils of Malaya having a pH above 6.0. Gaw (26) found Azotobacter present in 78 percent of the soils of Szechuei province of China.

The soils of East Africa were found to be almost devoid of Azotobacter (58), only 1 out of 40 soils samples being positive. Az. indicum (Beijerinckii Derx) was isolated from only one sample.

Kaila (47), studying Finnish soils, detected Azotobacter in only 5 out of 72 soils studied. Previous incubation of the soil with nutrients increased this number.

Jones and Murdock (46) isolated Azotobacter from 9 out of 17 soil types studied and concluded that they are present in most of the loams of Ontario. Lothead and Thexton (54), working with the soils of Ontario, found that Azotobacter were consistently higher in unfertilized soils than in fertilized, the numbers of Azotobacter showing no correlation with the productivity of the soils. Milne (59) found no difference in nitrogen-fixation in cultures inoculated with virgin and cultivated soils of Saskatchewan.

No difference was found in the Azotobacter population of cultivated, virgin, and non-irrigated soils of Washington. The Azotobacter population in any of the non-irrigated semi-arid soils tested was believed to be too small to contribute significantly to nitrogen fixation (74). A survey of 283 Colorado soils showed that 74 percent contained Az. chroococcum and 7 percent Az. vinelandii (28). No Azotobacter were found in 26 percent of the soils. The organisms were more widely distributed in irrigated than in dry soils.

Eseltine (22) found 100 percent occurrence of Azotobacter in South Carolina soils tested immediately after sampling. Drying of the soil resulted in a lower percentage recovery of these organisms.

Waksman (76) attributed the spasmodic occurrence of Azotobacter to the following factors:

1. Azotobacter usually cannot develop in a soil having a pH of less than 6.0.

2. The carbonate-phosphate ratio in soil was found to influence markedly the development of this organism.
3. Cropping, fertilization, and irrigation also affected the organisms. Higher counts were found in the fertilized and in the irrigated soils, than in the virgin or cultivated soils which had received no fertilizer or irrigation water.

Isolation and Study of Nitrogen-Fixing Organisms

Further research will probably uncover other organisms capable of fixing atmospheric nitrogen. Refinements of the standard methods now employed and more intense utilization of the special techniques involving special equipment, such as the mass spectrophotometer, radioactive isotopes, and the Warburg respirometer, will probably clarify some of the earlier unconfirmed reports regarding nitrogen fixation in such organisms as Aerobacter aerogenes (32).

The standard methods of the past have been:

1. The soil incubation method in which a quantity of soil, usually 100 gms., is mixed with 1 or 2 percent mannite. Sufficient water is added to bring the moisture to optimum condition.
2. The solution method in which 50 or 100 cc. portions of carbohydrate containing nitrogen-deficient mineral solution in Erlenmeyer flasks is inoculated with a small amount of soil (15). This method has been modified by the addition of white quartz sand resulting in better aeration and consequent nitrogen fixation (59). Bubbling of sterilized air through the media is also advantageous (15).

3. The Winogradsky method (15) includes the 5 determinations listed below:

- A. Silica gel plates 9 cm. in diameter are inoculated with 50 soil granules. Colonies developing on the plates are counted after 48 hours.
- B. One-half gm. of mannite is mixed with 50 gm. of moist soil. A microscopic ~~deter~~mination is made after 48 hours.
- C. Five percent starch is mixed with the moist soil, then molded into petri dishes. The colonies appearing on the smooth surface of the soil are counted after 48 hours.
- D. Twenty cm. silica gel plates are inoculated with 1 gm. of soil and colonies counted after 48 hours.
- E. The nitrogen fixed on the 20 cm. plates is determined after 5 days incubation.

The soil incubation method and the solution method are criticized by Currie (15). Only small amounts of nitrogen are fixed, resulting in a lack of significant comparisons. Azotobacter is forced to compete with many other organisms for the energy provided. Any toxic substances produced during incubation accumulate and may retard Azotobacter activity.

Currie (15) recommends the use of aluminum petri dishes filled with a mannitol solution of 1.5 percent agar instead of silica gel. Soil granules 20 to 40 mesh in size are inoculated onto 8 replicate plates. The nitrogen content of the medium is determined following incubation and a colony count is made. By this method it was found that the nitrogen fixed per colony is inversely related to the logarithm of the number of colonies on the plate.

Bryan (8) recommends the use of capped colonies on mannite agar plates containing a 1/40,000 dilution of the dye, congo red. Aleem (1) has suggested the use of sodium benzoate for isolating and identifying Azotobacter. Azotobacter utilizes sodium benzoate readily, resulting in very black characteristic colonies. Other organisms are depressed (64).

MATERIAL AND METHODS

Collection of Samples

Soil samples to be used for the study of the occurrence of aerobic, non-symbiotic nitrogen-fixing organisms were taken from various zones of Alberta.

To determine the effects of cropping, samples were taken from adjacent cropped and virgin soils. In areas affected by irrigation, virgin, cultivated non-irrigated, and cultivated irrigated soil samples were collected. The 0 - 6-inch depth of the cultivated and virgin soils was sampled aseptically in duplicate. This procedure was then repeated with the 6 - 12-inch depth.

Sampling equipment consisted of: sterilized 500 ml. wide-mouthed Erlenmeyers plugged with absorbent cotton, a six-inch spade, a one-inch steel gouging chisel, a spatula, and a propane torch for sterilization purposes. The gouging chisel was found necessary for sampling some of the hard solonchic soils. It proved a good tool for all sampling purposes where maintaining sterile conditions was necessary.

As illustrated in Figure 1, samples were taken in the following zones.

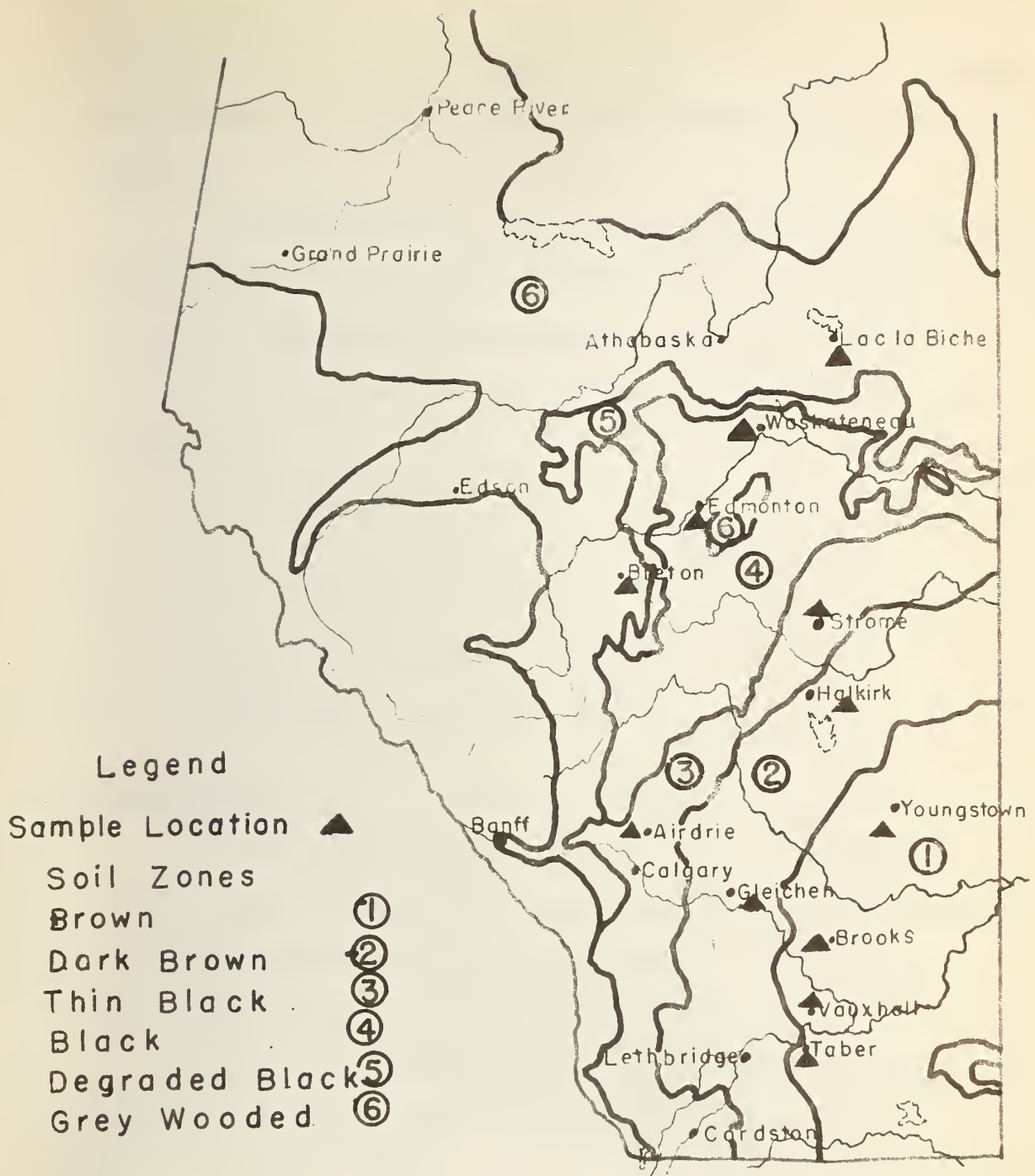


Fig. 1—Sketch map of central and southern Alberta showing location of soil samples.

Brown Soil Zone:

This semi-arid, short grass prairie area has an annual precipitation of 11 - 13 inches. The soils in this area are generally low in nitrogen and under irrigation often respond to phosphorus fertilizers.

The Taber, Vauxhall, and Brooks areas have extensive irrigation systems. Therefore soil samples were taken from the dry virgin, cultivated non-irrigated and the cultivated irrigated soils of this area.

The Youngstown samples consisted of virgin and cultivated dry land soils.

Sample locations:

Taber	NW 29 - 9 - 16 - 4 (Chin*)
Vauxhall	E $\frac{1}{2}$ 25 - 13 - 16 - 4 (Chin*)
Brooks	S $\frac{1}{2}$ 18 - 19 - 14 - 4
Youngstown	SW 27 - 28 - 10 - 4

Dark Brown Soil Zone:

The average annual precipitation is 13 to 15 inches and there are fewer droughts in this zone than in the brown. Although it is still designated as a short grass prairie, the grass makes a denser cover and a taller growth than in the Brown Zone.

Soils of this area are slightly higher in nitrogen and organic material than are the soils of the Brown Zone.

Sample locations:

Gleichen	SW 17 - 23 - 22 - 4
Halkirk	NE 12 - 38 - 15 - 4 (Halkirk*)

* Series name.

Thin Black:

Grassland with bluffs of trees in moist areas characterizes the vegetation of this zone. The soils are usually moderately high in nitrogen and organic matter if exhaustive cropping has not depleted the supply.

Sample locations:

Strome SE 25 - 44 - 15 - 4 (Killam loam*)

Airdrie SW 4 - 27 - 1 - 5

Black Soil Zone:

The annual precipitation of 17 to 19 inches and the lower evaporation rates have resulted in a parkland vegetation of grassland partially invaded by trees. This is the most fertile zone in the province, the nitrogen and organic matter content being 3 or 4 times that of the Brown Zone.

Sample locations:

Edmonton Series C plots, University of Alberta, Soil Science
Department (Malmo*)

Waskatenau NW 11 - 59 - 19 - 4

Grey Wooded Soil Zone:

Cooler temperatures and an annual precipitation of 12 to 20 inches have produced a mixed deciduous evergreen woodland in which bogs are of common occurrence. The soils in this zone are relatively less fertile because of leaching. They are low in nitrogen and organic matter and frequently deficient in sulphur and phosphorus.

Sample locations:

Lac La Biche NW 35 - 66 - 14 - 4

Breton, Series F plots, Soil Science Departmental Experimental Plots.
(Breton loam*)

* Series name.

The samples collected in the summer of 1954 were allowed to air-dry in the Erlenmeyers. After drying, the soils were ground with a mortar and pestle that had been sterilized by dry heat. The samples were then returned to sterilized Erlenmeyers. The samples collected in the summer of 1955 were not dried before use.

The reactions (pH) of the samples were determined using a Beckmann pH meter (Model N).

Isolation and Study of Aerobic Non-Symbiotic Organisms

Capable of Fixing Atmospheric Nitrogen

Reagents Employed

Gram's stain, Hucker Modification (56).

Ammonium oxalate crystal violet.

Gram's modification of Lugol's solution.

Safranin counterstain.

Ziehl's Carbol Fuchsin. Emended statement of Formula (56).

Loeffler's alkaline methylene blue.

Methylene blue in dilute alcohol.

Ehrlich's reagent (56).

Nutrient broth.

Nutrient agar.

Peptone purity control medium (12).

1% peptone.

.1% meat extract.

1.1 gm. mineral salt mixture.

1,000 ml. H₂O.

Mineral Salt Mixture

Grams of mineral salts per litre of distilled H₂O.

Dipotassium phosphate	K ₂ HPO ₄	0.5
Magnesium sulphate	Mg ₂ SO ₄	0.2
Sodium chloride	NaCl	0.2
Calcium sulphate	CaSO ₄	0.1
Ferrous sulphate	FeSO ₄	0.01
Sodium molybdate	Na ₂ MoO ₄ ·7H ₂ O	0.05
Manganese sulphate	MnSO ₄ ·H ₂ O	0.05

The essential salts in the proportions given above were ground in a mortar and stored for use.

Mannitol agar medium.

1.1 gm. salt mixture.

20 gm. of mannitol (C₆H₁₄O₆).

15 gm. Bacto agar.

1,000 ml. of water.

For the mannitol solutions, the agar was not added to the above medium.

Sodium benzoate agar medium.

1.1 gm. salt mixture.

1 gm. sodium benzoate (C₆H₅O₂Na).

15 gm. Bacto agar.

1,000 ml. of water.

Congo red mannitol agar medium.

A 1/40,000 dilution of the dye, congo red, was added to the mannitol agar medium.

Potassium nitrate mannitol agar medium.

Mannitol agar medium plus 0.1% KNO_3 .

Mannitol starch agar medium.

Mannitol agar medium plus .2% soluble starch.

Starch agar medium.

1.1 gm. salt mixture, 20 gm. soluble starch, 15 gm. agar
plus 1,000 ml. of distilled water.

Gelatin Liquifaction medium.

Gelatin tubes.

12% gelatin in H_2O , pH adjusted to 7.0.

Mannitol gelatin agar medium.

.4% gelatin added to mannitol agar medium.

Kligers iron agar medium (56).

Sugar and alcohol cleavage medium.

Mannitol, sucrose, lactose, and glucose broths in Durham fermentation
tubes with Vandredii's indicator.

Litmus milk (56).

To tubes of sterile skim milk enough saturated sterile, lime-free
litmus solution was added to give a lavender color.

Methods of Isolation of Aerobic Non-Symbiotic

Nitrogen-Fixing Organisms

Congo Red Mannitol Agar Plates

An attempt was made to study the nitrogen organisms of the
soil by using Bryan's capped colony technique (8). One ml. of a one
to ten dilution of the soil under study was inoculated onto mannitol
agar plates containing congo red. The plates were then capped with

another 5 ml. of medium and incubated. This method, in which the implanted Azotobacter colonies were to develop as pink sub-surface colonies, did not prove feasible on testing and was discarded because the small colonies that developed could not be aseptically isolated for pure culture study.

Mannitol Agar and Sodium Benzoate Agar Dust Plates

One-tenth of a gram of pulverized soil was shaken from a spatula onto a petri dish containing mannitol agar. Duplicate plates were used. The plates were incubated for 7 days at 28°C. then examined for growth.

Any growth that developed was stained with Gram's stain and examined microscopically. A similar procedure was used for isolating nitrogen-fixing organisms on plates of sodium benzoate agar.

Inoculation into Mannitol Solution

Twenty-five ml. of mannitol water were placed in 250 ml. Erlenmeyer flasks, stoppered with absorbent cotton and sterilized. The flasks were then inoculated with approximately 0.1 gm. of soil.

After an incubation period of 21 days the cultures were examined for visible signs of growth; surface film, cloudiness, color or odor. Microscopic examinations were also conducted before inoculating mannitol and sodium benzoate plates with this culture. The plates were examined for growth after 7 days' incubation and further inoculations made to isolate and purify the nitrogen-fixing organisms.

Microscopic Examination

Detailed microscopic examinations were conducted on organisms during the course of the investigation. This was done to check for

any variations in the morphology of the cells and to maintain the purity of the cultures.

The cell size determination was made by using a calibrated eyepiece. The motility of the organisms was determined by making hanging drop preparations from liquid and solid culture media at different times and conditions of incubation.

Growth Characteristics

The macroscopic growth characteristics and the microscopic morphological features were determined using pure cultures of the organisms capable of fixing atmospheric nitrogen. The media employed for this study consisted of: nutrient broth, nutrient agar plates and slants, sodium benzoate agar plates and slants, peptone water and gelatin stabs.

The effect of small concentrations of nitrate were determined by the inoculation of the 0.1% potassium nitrate mannitol agar plates.

For pure culture studies of the organisms all tests were made in duplicate. All the tests except the production of indol and the reduction of litmus milk were also repeated. This resulted in two separate duplicate determinations. The determination of the nitrogen-fixing ability of the organisms was done with four replicates and was not repeated. In all cases unless otherwise specified, the incubation period was 7 days at 28°C.

Starch Hydrolysis

The ability of the microorganisms to develop with starch as the only source of energy was determined by using starch agar plates. The hydrolysis of starch was also determined by making single streak

inoculations on starch mannitol agar. After incubation the plates were flooded with Lugol's iodine. Absence of the typical blue starch-iodine color indicated starch hydrolysis in the presence of mannitol.

Gelatin Liquifaction

Gelatin liquifaction was determined by streak cultures on 0.4% gelatin mannitol agar plates. After incubation the plates were covered with 10 ml. of a solution of 15 gm. of HgCl_2 in 100 ml. of distilled water and 20 ml. of concentrated HCl. The reagent formed a white opaque precipitate with the unchanged gelatin. Organisms utilizing gelatin were surrounded by a clear zone.

Indol Production

Indol production was determined by growing the cultures in tryptophane broth. After four days incubation the cultures were mixed with 5 ml. ether. Then Ehrlich's reagent was layered between the ether and the culture medium. A red color appearing in five minutes indicated a positive reaction.

Production of Hydrogen Sulphide

The test strip technique was used. The organisms were grown in tubes of peptone broth equipped with lead acetate paper strips. Blackening of the strips indicated hydrogen sulphide production. Hydrogen sulphide was also determined by using tubes of Kligler's iron agar in which blackening of the red medium indicated the presence of hydrogen sulphide.

Cleavage of Sugars and Alcohols

Durham tubes containing sucrose, glucose, lactose, and mannitol were inoculated with the organisms and subsequently examined

the Board of Directors of the Corporation, and the Corporation is authorized to execute and deliver the same, and to do all such acts and things as may be necessary or proper to carry out the purposes of the foregoing.

Section 10. Amendment.

This Certificate of Incorporation may be amended or altered by the affirmative vote of a majority of the outstanding shares of the Corporation, and any such amendment or alteration shall be binding upon the Corporation and its shareholders as if it were contained in this Certificate of Incorporation. The Board of Directors of the Corporation is authorized to execute and deliver the same, and to do all such acts and things as may be necessary or proper to carry out the purposes of the foregoing.

Section 11. Dissolution.

The Corporation shall continue in existence until the expiration of the term for which it was organized, and until the affirmative vote of a majority of the outstanding shares of the Corporation shall be taken to dissolve the Corporation. The Board of Directors of the Corporation is authorized to execute and deliver the same, and to do all such acts and things as may be necessary or proper to carry out the purposes of the foregoing.

Section 12. Successors.

The Corporation shall continue in existence until the expiration of the term for which it was organized, and until the affirmative vote of a majority of the outstanding shares of the Corporation shall be taken to dissolve the Corporation. The Board of Directors of the Corporation is authorized to execute and deliver the same, and to do all such acts and things as may be necessary or proper to carry out the purposes of the foregoing.

Section 13. Governing Law.

This Certificate of Incorporation shall be governed by the laws of the State of New York. The Board of Directors of the Corporation is authorized to execute and deliver the same, and to do all such acts and things as may be necessary or proper to carry out the purposes of the foregoing.

for acid and gas production.

Litmus Milk Reduction

Tubes containing litmus milk were inoculated and examined after 14 days incubation.

Effect of Reaction (pH)

The ability of the non-symbiotic nitrogen-fixing organisms under study to grow at various pH levels was determined. The mannitol agar medium was adjusted to the proper pH range by the addition of 0.05 M. KH_2PO_4 and enough 0.5 M. NaOH to get the desired reaction. Below a pH of 5.8 the medium was unbuffered, HCl being used to obtain the desired pH. Growth at pH's below 5.8, in which the KH_2PO_4 - NaOH buffer is not effective, was also tested by using a phthalate - NaOH buffer pair.

Optimum Temperatures of Growth

Mannitol agar tubes were inoculated, then incubated at temperatures of 4°C., 8°C., 18°C., 25°C., 28°C., and 37°C. The tubes were examined for growth after 3 days, 7 days, and, in the case of the colder temperature, 21 days incubation.

Thermal Death Point

Melted mannitol agar tubes were placed in a water bath at temperatures of 50°C., 55°C., 60°C., 65°C., and 70°C., respectively. The tubes of media were allowed to attain the temperature of the bath. Then the tubes were inoculated with a heavy suspension of the organisms under study. The tubes were left in the bath another 10 minutes, then removed and immediately poured into previously chilled petri dishes. After incubation the plates were examined for growth.

Nitrogen Fixation

The nitrogen-fixing ability of the organisms was determined by inoculating 2 ml. of a suspension of washed resting cells into a buffered liquid solution containing mannitol as the energy source. The medium was buffered by using 1.8 gm. K_2HPO_4 and 0.7 gm. of KH_2PO_4 in 1,000 ml. of water plus the other essential mineral salts as used in the mannitol solution.

The use of this buffered pair resulted in a buffered nutrient solution of pH 7.2.

The appendix describes in detail the methods and conditions of inoculation and incubation of the nitrogen-fixing organisms under study. The method of determining the nitrogen-fixing ability by using a microkjeldahl nesslerization procedure is also discussed.

RESULTS AND DISCUSSION

Isolation and Identification of Aerobic Non-Symbiotic Nitrogen-Fixing Organisms

The results obtained by the inoculation of the mannite and sodium benzoate dust plates are presented in Table I. The results for the brown soil zones only are presented. There was no discernable growth on any of the plates except those inoculated with Waskatenau and Lac La Biche soils where minute colonies were discernable. The occurrence of Azotobacter in Alberta soils, as determined by the mannite agar and sodium benzoate agar dust plate techniques, is closely correlated with irrigation practices in this province.

Table II presents the results obtained by inoculating a mannitol solution with 0.1 gm. of soil obtained from the irrigated, cultivated non-irrigated, and virgin soils of some of the irrigation districts in Alberta.

Examination of the mannitol solution for scum, sediment, turbidity, gas production and odor in conjunction with detailed microscopic examination showed typical yeastlike Azotobacter to be present only in the cultivated-irrigated soils of Taber and Vauxhall.

The dust plates failed to show the presence of typical Azotobacter in the cultivated non-irrigated soils of Taber and Vauxhall. This is probably because the organisms were present in much smaller numbers in the non-irrigated than in the irrigated areas. Typical Azotobacter were not found in the virgin soils of Alberta by any of the isolation methods used.

The primary difference in results between the dust plate methods, in which soil is sprinkled onto solid agar, and the mannitol solution method, is found in the non-irrigated soils of Alberta.

The results for the mannitol solution method with subsequent inoculation onto mannitol and sodium benzoate agar are presented in Table III. Microscopic examination of the mannitol solutions after incubation showed that, although no scum was formed on the surface of the medium, in the majority of cases a small Gram negative coccoid rod was present. This organism did not stain well with either Gram's or methylene blue stain, tending to stain only at the edges.

Inoculation of mannitol agar plates from liquid cultures gave rise to large brownish-grey, mucilaginous colonies on some of the plates. All the soils in Alberta contained an organism producing small 1 - 2 mm. unpigmented circular, entire, lens-shaped colonies.

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Sodium benzoate plates inoculated with irrigated soils gave rise to 3 - 4 mm. black, flat circular colonies. All of the soils sampled also developed 1 - 2 mm. entire circular colonies on this medium. These colonies were unpigmented except for the Lac La Biche cultivated and virgin, and Breton cultivated soils, in which cases the growth had a dark brown soluble pigment.

Microscopic examination of the mannitol agar and sodium benzoate agar plates as recorded in Tables II and III corroborated the results obtained in the examination of the liquid cultures.

The handling of replicate plates for duplicate samples of soil from the different depths and cropping practices for each of the soils studied entailed a great deal of routine work. To facilitate the isolation, study and characterization of the organisms involved, attempts were made to reduce the number of cultures handled. The cultures were grown on mannitol and sodium benzoate agar slants, nutrient agar plates and slants, and in nutrient broth and peptone media.

The results of these tests together with the data presented in Tables II and III were used to reduce the number of organisms studied down to one of the smaller organisms for each of the soils sampled and one representative of the typical Azotobacter found in each of the southern irrigated soils. Although the smaller non-pigment producing organisms were also isolated from the Brooks soil, they were not studied in pure culture.

This resulted in 14 cultures being used for the study and characterization of the aerobic non-symbiotic nitrogen-fixing organisms isolated from Alberta soils studied in this investigation. Table IV presents the name given to the various cultures under study and the location from which each organism was isolated. The term light (Lt.) was used to designate the small unpigmented cells in soils from which more than one type of organism was isolated.

TABLE I. INITIAL ISOLATION OF NON-SYMBIOTIC NITROGEN-FIXING ORGANISMS
ON MANNITOL AGAR AND SODIUM BENZOATE DUST PLATES

Location of Soil Sampled	Cropping Practice	Sampling Depth, In.	Growth on Agar Plates Inoculated With 0.1 gr. Soil	
			Mannitol Agar	Sodium Benzoate Agar
Taber	Cult. Irrig.	0 - 6	3 large irregular light brown col.	2 black raised col. 3 - 4 mm. diam.
		6 - 12	1 large irregular light brown col.	1 black raised col. 1 - 2 mm. diam.
	Cult. non Irrig.	0 - 6	No visible growth	No visible growth
		6 - 12	No visible growth	No visible growth
	Virgin	0 - 6	No visible growth	No visible growth
		6 - 12	No visible growth	No visible growth
	Cult. Irrig.	0 - 6	3 large irregular light brown col.	3 black circular raised col. 3 - 4 mm. diam.
		6 - 12	1 large irregular light brown col.	1 black circ. raised col. 3 - 4 mm. diam.
Vauxhall	Cult. non Irrig.	0 - 6	No visible growth	No visible growth
		6 - 12	No visible growth	No visible growth
	Virgin	0 - 6	No visible growth	No visible growth
		6 - 12	No visible growth	No visible growth
Brooks	Cult. Irrig.	0 - 6	3 large irregular brown flat col.	2 black circular raised col.
		6 - 12	1 large irregular brown flat col.	1 black circular raised col.
	Cult. non Irrig.	0 - 6	No visible growth	No visible growth
		6 - 12	No visible growth	No visible growth
	Virgin	0 - 6	No visible growth	No visible growth
		6 - 12	No visible growth	No visible growth
Youngstown	Cult. non Irrig.	0 - 6	1 - 2 mm. entire milky col.	No visible growth
		6 - 12	1 - 2 mm. entire milky col.	No visible growth
	Virgin	0 - 6	1 - 2 mm. entire milky col.	No visible growth
		6 - 12	1 - 2 mm. entire milky col.	No visible growth

TABLE II. ISOLATION AND IDENTIFICATION OF NON SYMBIOTIC NITROGEN FIXING ORGANISMS OF IRRIGATED AREAS IN ALBERTA

Soil Sampled	Cropping Practice	Sampling Depth, In.	pH	Examination of Growth in Mannitol Soln.		Mannitol Agar Inoculated From Mannitol Soln.		Sodium Benzoate Agar Inoculated from Mannitol Soln.	
				Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic
Taber	Cultivated Irrigated	0 - 6	7.8	Brown scum, gas produced, turbid liquid	2 x 4 u. yeastlike cocci + 1 x 2 u. coccoid rods	2-4 mm. col. yellow to brown and 1-2 mm. glistening. Unpigmented	2 x 4 u. heavily stained organisms plus 1 x 2 u. coccoid rods	2-3 ml. black col. plus 1-2 mm. milky glistening col.	2 x 4 u. rods plus 1 u. cocci
		6 - 12	7.7						
	Cultivated non Irrigated	0 - 6	7.7	Brown heavy scum, gas produced. No visible growth in 6 - 12"	2 x 4 u. yeastlike in 0-6". Small rods in both 0-6" and 6-12"	Heavy growth of brown col. 3-4 mm. in 0-6" plus smaller unpig.	2 x 3 u. yeastlike showing granules plus smaller 1 u. coccoid	0-6" showed black pigmented growth also 1 mm. milky col.	2 x 4 u. yeastlike plus 1 u. coccoid rods
		6 - 12	7.8						
	Virgin	0 - 6	7.9	No visible growth	Small coccoid rods 1 x 2 u.	Milky glistening 1 mm. circular convex col.	1 x 2 u. coccoid rods	1-2 mm. milky glistening col.	1 x 2 u. coccoid rods
		6 - 12	8.0						
Vauxhall	Cultivated Irrigated	0 - 6	7.4	Brown scum on turbid liquid. Gas produced	2 - 3 u. yeastlike cocci plus small .75 x 1.5 u. rods	Spreading brown mucous col. plus small unpigmented	2 - 3 u. yeastlike cocci and small coccoid rods	2-3 mm. black col. and small unpigmented ones	2 x 3 u. yeastlike cocci and small coccoid
		6 - 12	7.8						
	Cultivated non Irrigated	0 - 6	7.2	Heavy scum on 0-6". No visible growth on 6-12"	Yeastlike cocci in 0-6", 1 x .75 u. coccoid rods in 6-12"	1 mm. glistening circular unpigmented col.	1 u. coccoid resemble staphylococcus	1 mm. col. slight pigment on 0-6", none on 6-12"	1 x 2 u. coccoid rods
		6 - 12	7.5						
	Virgin	0 - 6	7.5	No visible growth	Small coccoid rods	1-2 mm. circular unpigmented col.	Small coccoid rods	1-2 mm. circular unpigmented col.	Small coccoid rods
		6 - 12	7.4						
Brooks	Cultivated Irrigated	0 - 6	7.4	Slight brown scum, mucilaginous sediment	2-3 mm. yeastlike cocci and small coccoid organisms	3-4 mm. yellow and 1-2 mm. unpigmented col.	2 x 4 u. rods and small coccoid organisms	Black 3-4 mm. col. in 0-6". 1-2 mm. unpig. in 6-12"	2 x 4 u. rods and small coccoid
		6 - 12	7.3						
	Cultivated non Irrigated	0 - 6	6.9	Slight sediment. No other signs of growth	.75 x 1 u. coccoid organisms, strain darker on outer edges	1 mm. milky circular col.	.75 x 1 u. coccoid organisms, stain darker on outer edges	1-2 mm. unpigmented circular entire col.	.75 x 1 u. coccoid organism
		6 - 12	6.6						
	Virgin	0 - 6	6.6	Slight sediment	Coccoid rods 1 x 1.25 u.	1 mm. unpigmented circular col.	1 x .75 u. coccoid rods	1 mm. unpigmented circular col.	Small coccoid rods
		6 - 12	6.8						

TABLE III. ISOLATION AND IDENTIFICATION OF NON-SYMBIOTIC NITROGEN-FIXING ORGANISMS OF CULTIVATED AND VIRGIN SOILS IN ALBERTA

Soil Sampled	Sampling Practice	Sampling Depth, in.	pH	Examination of Growth in Mannitol Solution		Mannitol Agar, Inoculated from Mannitol Solution		Sodium Benzoate Agar, Inoculated from Mannitol Solution	
				Macroscopic	Microscopic	Macroscopic	Microscopic	Macroscopic	Microscopic
Youngstown	Cult.	0 - 6	6.0	Light scum	1.5 x 2 u. rods	2 - 3 mm. milky	1 x 1.5 u. rods	1 - 3 mm. col.	1 x 1.5 u.
		6 - 12	7.6	Brown sediment	lightly stained	smooth col.	round ended	unpigmented	coccoid
	Virgin	0 - 6	6.1	Heavy scum	1.5 u. coccoid	1 - 3 mm. col. mu-	.75 x 1 u. rods	1 - 3 mm. col.	1 x 1.5 u.
		6 - 12	8.6	Slight sediment	lightly stained	cilaginous unpig.	round ended	unpigmented	coccoid
Gleichen	Cult.	0 - 6	7.8	Brownish	Small coccoid	1 - 2 mm. circular	.75 x 1 u. cocci	1 mm. unpig.	1 u. coc-
		6 - 12	8.1	sediment	rods	unpigmented col.	in clusters	col.	coid rods
	Virgin	0 - 6	7.0	Brownish	Small coccoid	1 - 2 mm. circular	.75 x 1 u. cocci	1 mm. unpig.	1 u. coc-
		6 - 12	8.6	sediment	rods	unpigmented col.	plus small rods	col.	coid rods
Halkirk	Cult.	0 - 6	4.6	No visible	2 u. cocci	1 - 3 mm. circular	1.5 x 2 u.	2 - 3 mm. unpig.	1.5 x 2 u.
		6 - 12	5.0	growth	showing granules	unpigmented col.	coccoid organism	circular col.	coccoid org.
	Virgin	0 - 6	4.8	Light	2 u. cocci	2 - 3 mm. circular	1.5 x 2 u.	2 - 3 mm. circular	1.5 x 2 u.
		6 - 12	6.9	sediment	showing granules	unpigmented col.	coccoid organism	unpigmented col.	coccoid org.
Strome	Cult.	0 - 6	5.6	No visible	1 x 2 u. rods	1 mm. unpig.	.75 x 1.5 u.	1 - 2 mm. unpig.	1 x 1.5 u.
		6 - 12	6.6	growth	stained edges	tear drop colony	coccoid rods	col.	clustered rods
	Virgin	0 - 6	5.8	Light	1 x 2 u. coccoid	1 mm. unpig.	.75 x 1.5 u.	2 - 3 mm. lens	1 x 2 u.
		6 - 12	6.2	sediment	rods stained edge	colony	coccoid rods	shaped unpig.	coccoid rods
Airdrie	Cult.	0 - 6	8.1	Very light	1 x 2 u. rods	2 - 3 mm.	1 x 2 u. stain	2 - 3 mm. unpig.	Rods may
		6 - 12	8.5	sediment	stained edges	circular col.	on outer edge	col.	be diploids
	Virgin	0 - 6	7.2	Grayish growth	1 x 2 u. coccoid	1 - 3 mm.	1 x 2 u. stain	1 mm.	Rods may
		6 - 12	6.8	plus sediment	rods stained edge	unpig. col.	on outer edge	unpig. col.	be diploids
Edmonton	Cult.	0 - 6	6.1	Slight	1 u. coccoid	1 mm.	.75 x 1 u.	1 - 2 mm.	1 x 2 u.
		6 - 12	6.0	sediment	organisms	circular unpig.	coccoid rods	unpig. col.	clustered rods
	Virgin	0 - 6	5.0	Slight	1 u. coccoid	1 mm.	1 u. coccoid	2 mm. unpig.	1 x 2 u.
		6 - 12	5.2	sediment	organisms	circular unpig.	organism	col.	stained edge
Wasketenau	Cult.	0 - 6	7.9	White scum	1 x 1.5 u.	1 - 2 mm.	1 u. cocci	1 mm.	1.5 u. coc-
		6 - 12	7.9	No sediment	coccoid organism	colorless	in clusters	milky col.	coid clustered
	Virgin	0 - 6	6.5	No apparent	2 x 4 u. cocci	1 - 2 mm.	1 u. cocci	1 mm.	1 u. coccoid
		6 - 12	5.8	growth	plus smaller rod	colorless	in clusters	milky col.	clustered
Breton	Cult.	0 - 6	6.4	White scum	1 x 2 u.	1 - 2 mm. circular	1 x 2 u.	1 - 2 mm. col.	1 x 2 u.
		6 - 12	6.3	Slight sediment	coccoid rods	unpigmented col.	coccoid rods	black pigment	coccoid rods
	Virgin	0 - 6	6.4	No visible	1 x 2 u.	1 - 2 mm. circular	1 x 2 u.	1 - 3 mm. col.	1 x 2 u.
		6 - 12	6.3	effect	coccoid rods	unpigmented col.	coccoid rods	unpigmented	coccoid rods
Lac La Biche	Cult.	0 - 6	7.6	White scum	1.5 u. yeast-	3 - 4 mm.	1 x 2 u. cocci	1 mm. brown	1.5 u.
		6 - 12	6.6	slight sediment	like cocci	milky col.	plus small rods	col.	coccoid rods
	Virgin	0 - 6	7.3	Flocculent	1.5 u. yeast-	2 mm.	1 x 1.5 u.	1 mm.	1.5 u.
		6 - 12	6.0	sediment	like cocci	milky col.	coccoid rods	brown col.	coccoid rods

TABLE IV. ORGANISMS ISOLATED AND CHARACTERIZED IN PURE CULTURE

Location of soil from which organisms were isolated			
Name of Culture	Soil	Cropping Practice	Depth Duplicate
Taber	Taber	Cultivated non-irrig.	0 - 6 B
Taber (Lt.)	Taber	Cultivated non-irrig.	0 - 6 B
Vauxhall	Vauxhall	Cultivated irrig.	0 - 6 B
Vauxhall (Lt.)	Vauxhall	Cultivated irrig.	0 - 6 B
Brooks	Brooks	Cultivated irrig.	6 - 12 A
Youngstown	Youngstown	Cultivated	0 - 6 A
Halkirk	Halkirk	Cultivated	6 - 12 B
Strome	Strome	Virgin	0 - 6 B
Airdrie	Airdrie	Cultivated	0 - 6 A
Edmonton	Edmonton	Cultivated	0 - 6 B
Waskatenau	Waskatenau	Cultivated	0 - 6 A
Lac La Biche	Lac La Biche	Cultivated	0 - 6 A
Breton	Breton	Virgin	6 - 12 A

Characterization of Aerobic Non-Symbiotic Nitrogen-Fixing Organisms in

Pure Culture

In an endeavor to maintain the freedom from contaminants throughout the course of the investigation, aseptic techniques were strictly followed. As a further control, growth was observed microscopically as well as macroscopically in sugar-free peptone media in conjunction with most of the tests performed.

Growth and Morphology on Laboratory Media

Plate 1 shows the characteristic growth of the large typical Azotobacter on mannitol agar. Fig. A shows the browning of the circular smooth edged flat colonies, while Fig. B depicts the larger coalescent colonies with less pigment which are sometimes found after prolonged growth on nitrogen-free media.

The cultural characteristics of the smaller organisms isolated from all of the soils in Alberta are shown in Plate 2. These punctiform, convex, 1 mm. mucilaginous colonies were unpigmented on mannitol agar. On sodium benzoate agar the Lac La Biche and Breton colonies had a dark brown soluble pigment.

Mannitol agar slant colonies of the small organisms were beaded to filiform whereas the larger Azotobacter produced an extensive greyish-brown filiform growth. The typical Azotobacter did not develop on nutrient agar, in nutrient broth or in peptone water. The smaller coccoid rods grew on nutrient agar producing 2 - 3 mm., entire, circular, flat, white colonies. The growth on nutrient agar slants was white and filiform. Inoculation of nutrient broth produced a flocculent growth whereas peptone water became cloudy and had a slight ring on the surface.

PLATE 1. CULTURAL CHARACTERISTICS OF AZOTOBACTER GROWN ON MANNITOL AGAR



Figure A. Azotobacter isolated from Taber soils showing brown non-coalescent colonies.

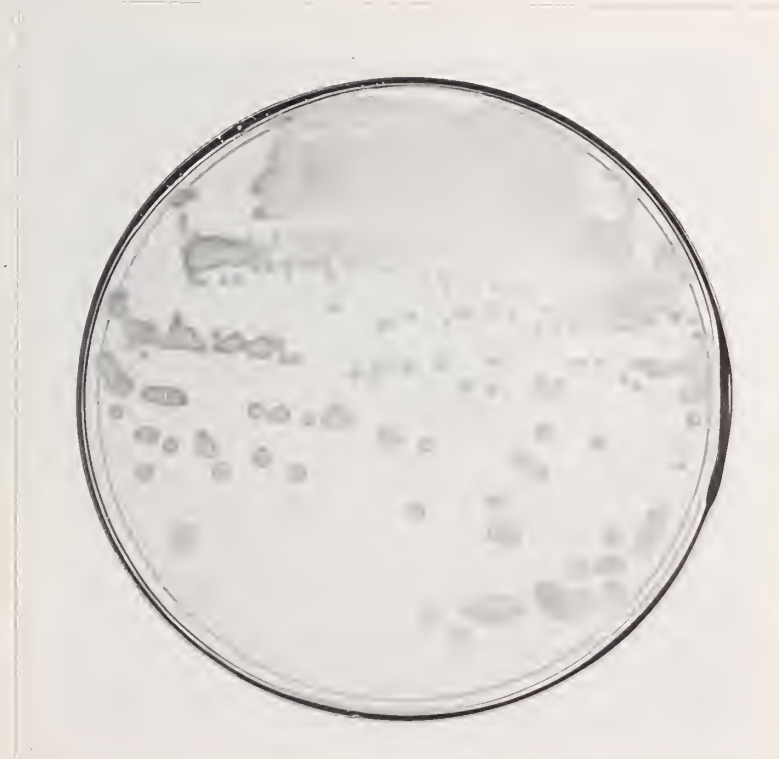


Figure B. Azotobacter isolated from Brooks, showing spreading coalescent colonies with less pigment.

PLATE 2. CULTURAL CHARACTERISTICS OF SMALL NITROGEN-FIXING ORGANISMS
ISOLATED FROM ALBERTA SOILS



Figure A. Nitrogen-fixing organisms isolated from Strome growing as unpigmented punctiform colonies.



Figure B. Nitrogen-fixing organisms isolated from Edmonton.

Microscopic Examination

The organisms under study were Gram-negative. Gram-positive inclusions were noticed on several occasions. The typical Azotobacter stained well with Gram's stain but the smaller organisms were difficult to stain with Gram's basic fuchsin or methylene blue. The smaller organisms tended to stain at the edges resulting in "halo and safety pin" effects.

Either Gentian violet or Ziehl's carbol fuchsin were found satisfactory for staining purposes. In this study the carbol-fuchsin was employed for the routine examination of the organisms. Plates 3 and 4 show the morphological characteristics of the typical Azotobacter isolated from the irrigation areas of Alberta. Plate 3 depicts the organisms in their predominant form. The plump rods measuring approximately 2 by 4 u. varied somewhat in size and shape. Occasionally longer cells up to 7 u. in length were found. The refractive granules as seen in Fig. B of Plate 3 and Fig. C and D of Plate 4 were found in all the typical Azotobacter isolated.

Fig. A and B of Plate 4 depict what research workers have called giant cells of Azotobacter.

Plate 4, Fig. D, depicts what has been called the disintegration of Azotobacter. This phenomenon was used by Bisset and Hale (7) in their arguments for reproduction of Azotobacter by gonidia. These various morphological forms were found in all the large typical Azotobacter cultures studied. They appear to be a natural part of the life cycle of this organism rather than characteristics of a different strain or of contaminants.

MORPHOLOGY OF AEROBIC NON-SYMBIOTIC NITROGEN-FIXING ORGANISMS*

PLATE 3 Cells of Azotobacter Stained with Carbol-Fuchsin.

- Fig. A. Organism from Taber growing as large plump rods.
- Fig. B. Plump rods showing refractive granules.
- Fig. C. Azotobacter occurring as 2 by 3 u. yeastlike organisms showing the tendency to occur in pairs.
- Fig. D. Yeastlike cocci tending to go to giant cells.

PLATE 4 Cells of Azotobacter Stained with Carbol-Fuchsin and Methylene Blue.

- Fig. A. Yeastlike Azotobacter occurring in pairs with a tendency to go to giant cells.
- Fig. B. Giant cells of Azotobacter showing disintegration.
- Fig. C. Methylene blue stain of Azotobacter showing refractive granules.
- Fig. D. Methylene blue stain of Azotobacter showing refractive granules of yeastlike cocci and larger cells.

PLATE 5 Non-Symbiotic Nitrogen-Fixing Organisms Isolated from Strome Soil.

- Fig. A. Organisms showing round ended rods which sometimes appear diploid, described on the initial isolation as .75 to 1 u. by 1.5 to 2 u. rods tending to be clustered.
- Fig. B. The same organism as in Fig. A showing up as smaller rods, tending to diploid and irregular grouping.
- Fig. C. Strome organism upon initial isolation showing clustering into an irregular pattern.

* All figures x 1870.

PLATE 6 Non-Symbiotic Nitrogen-Fixing Organisms Isolated From the
Brown and Dark Brown Soil Zones of Alberta.

- Fig. A. Organisms isolated from Youngstown soil.
Fig. B. Coccoid rods from the Gleichen area.
Fig. C. The 1 by 1.5 u. coccoid rods isolated from Halkirk soil.
Fig. D. Larger yeastlike organisms appearing in the Halkirk
cultures.

PLATE 7 Non-Symbiotic Nitrogen-Fixing Organisms Isolated from the
Black and Grey Wooded Soil Zones of Alberta.

- Fig. A. Typical coccoid rods tending to be diploids isolated
from Edmonton soil.
Fig. B. Nitrogen-fixing organisms isolated from Breton grey wooded
soil.
Fig. C. Nitrogen-fixing organisms isolated from soil in the
Lac La Biche district.

PLATE 3. MICROSCOPIC MORPHOLOGY OF TYPICAL AZOTOBACTER CELLS STAINED
WITH CARBOL FUCHSIN



Figure A.

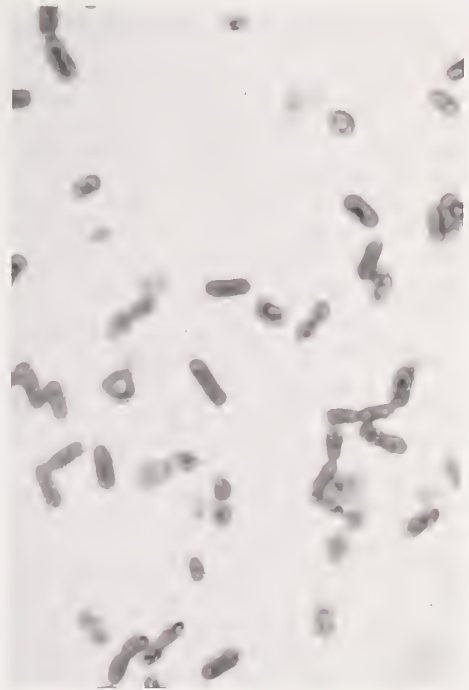


Figure B.

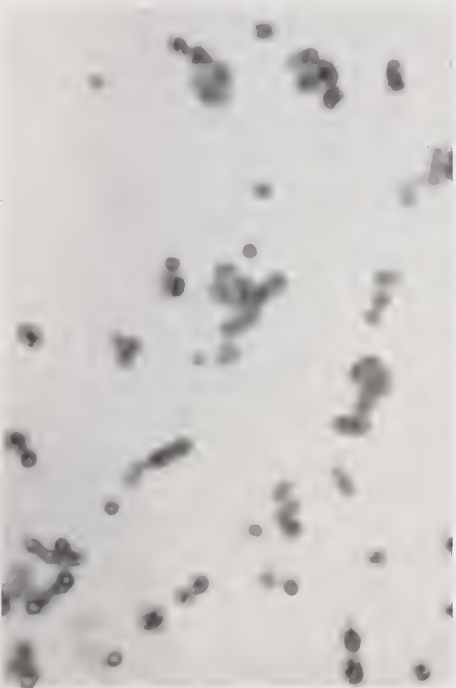


Figure C.

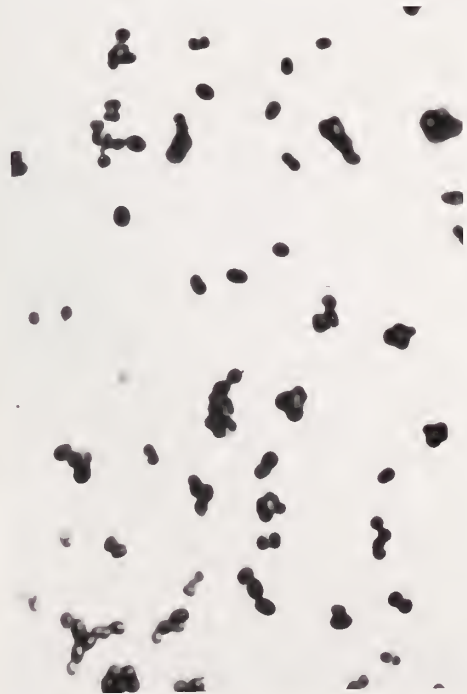


Figure D.

PLATE 4. CELLS OF AZOTOBACTER STAINED WITH CARBOL FUCHSIN AND
METHYLENE BLUE

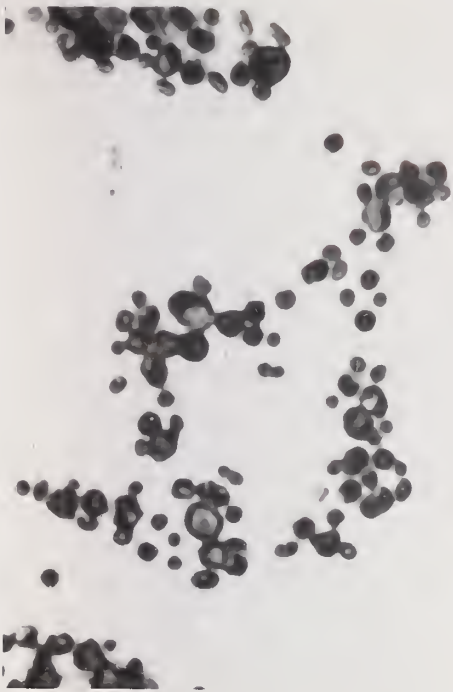


Figure A.

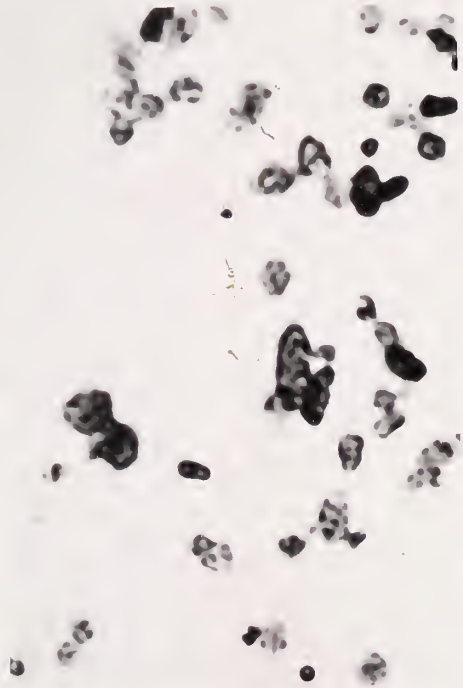


Figure B.

Carbol fuchsin

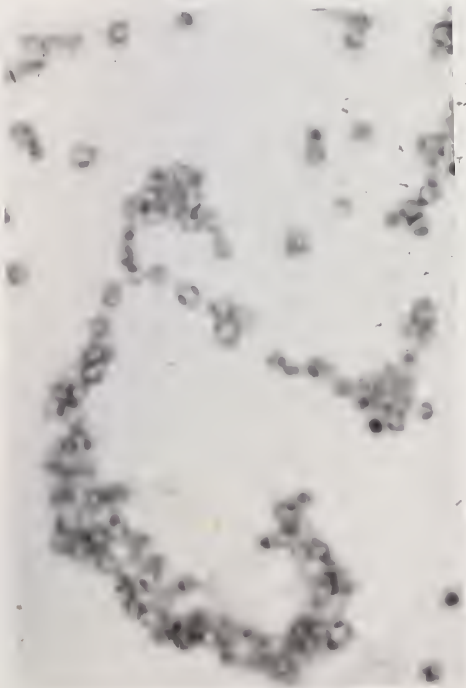


Figure C.

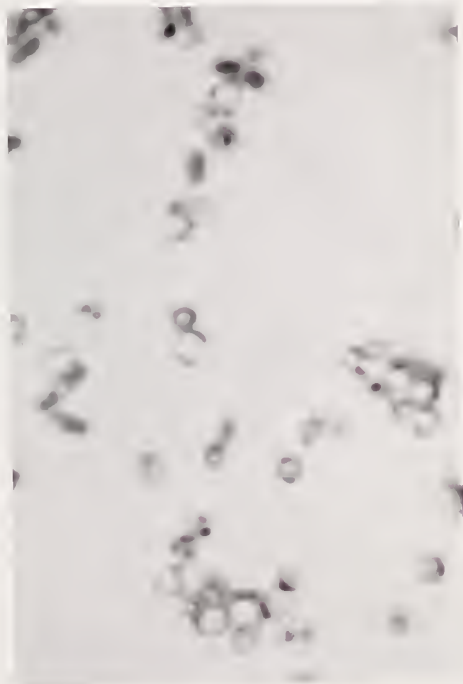


Figure D.

Methylene blue

PLATE 5. NON-SYMBIOTIC NITROGEN-FIXERS ISOLATED FROM STROME SOIL

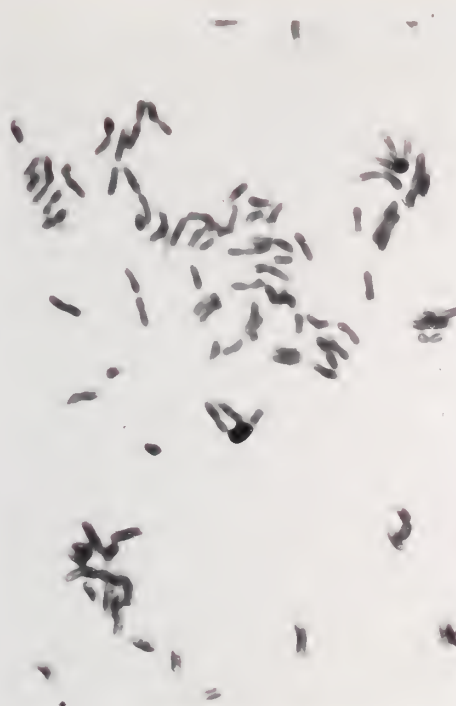


Figure A.



Figure B.



Figure C.

PLATE 6. NON-SYMBIOTIC NITROGEN-FIXING ORGANISMS ISOLATED FROM THE
BROWN AND DARK BROWN SOIL ZONES OF ALBERTA



Figure A.



Figure B.

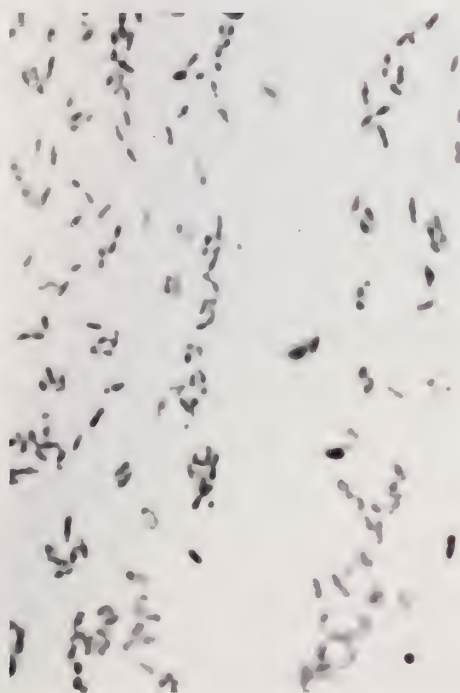


Figure C.



Figure D.

PLATE 7. NON-SYMBIOTIC NITROGEN-FIXING ORGANISMS ISOLATED FROM THE
BLACK AND GREY WOODED SOIL ZONES OF ALBERTA

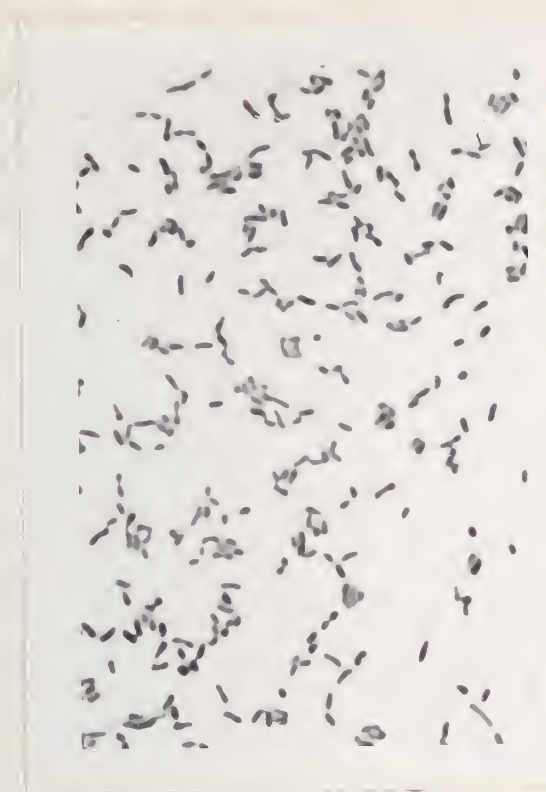


Figure A.



Figure B.

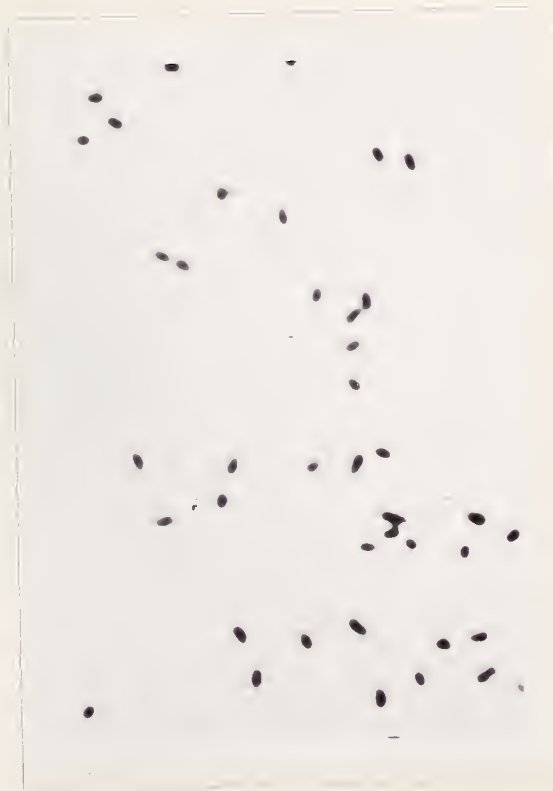


Figure C.

The study of the morphology of the smaller nitrogen-fixing organisms isolated from all of the soils in Alberta presented more difficult problems. On initial isolation, the majority of the organisms were described as 0.75 by 1.0 u. to 1.5 by 2.0 u. coccoid rods, having a tendency to appear as diploids in some cases. The cells were also observed in clusters and irregular groupings. Plates 5, 6, and 7 depict the microscopic morphology of these organisms. Some variation occurred in the size and shape of the cells as seen in these plates. However, these variations were not great enough to differentiate the organisms in microscopic examination studies.

Motility tests were conducted. The typical Azotobacter were definitely motile. As determined by the hanging drop method, Youngstown, Lac La Biche, and Taber (Lt.) were also motile. Motility was not observed in the remaining cultures.

Biochemical Properties

Growth on nitrate agar:

The cultures were grown on 0.1 percent potassium nitrate-mannitol agar to determine the effects of small concentrations of nitrates. Waskatenau, Breton, Airdrie, Lac La Biche, Youngstown, Cleichen, Halkirk, and Vauxhall (Lt.) grew profusely producing 3 - 4 mm. unpigmented colonies.

Taber (Lt.) and Edmonton did not grow as extensively, producing 2 - 3 mm. colonies with a light yellow tinge. Taber and Vauxhall produced 1 mm. brown pigmented colonies while the Brooks colonies were unpigmented. Nitrate appears unnecessary for the growth of typical Azotobacter cultures. It even inhibited the growth somewhat. However, it enhanced the growth of all the smaller organisms.

Utilization of starch:

All of the organisms were capable of growth when soluble starch was substituted for mannitol as the energy source. To determine the extent of hydrolysis of starch by the nitrogen-fixing organisms, 0.2 percent starch was incorporated into mannitol agar and inoculated with streaks of cultures. Upon flooding with Lugol's iodine, the unused starch in the medium turned blue. The extent of starch hydrolysis was indicated by a clear zone not having the starch-iodine color around the colonies. The typical Azotobacter cultures utilized starch extensively. All the other organisms, although not using as much starch, definitely showed hydrolysis.

Gelatin Liquifaction:

Gelatin stab cultures did not show any liquifaction. The plate method of determining gelatin liquifaction also gave negative results.

Indol production:

None of the aerobic non-symbiotic nitrogen-fixing organisms isolated from Alberta soils produced indol from tryptophane.

Hydrogen sulphide production:

Table V presents the results for the tests conducted to determine if the cultures under study produce hydrogen sulphide. The typical Azotobacter do not produce hydrogen sulphide, whereas the smaller nitrogen-fixing organisms appear to be positive for this test. Organisms isolated from Edmonton and Taber (Lt.) are exceptions and did not produce hydrogen sulphide under the condition of the tests. For the smaller organisms the lead acetate strip technique did not show as many positive results as did the Kligler's. It is not known whether this was due to a difference in sensitiveness of the two tests or whether different organisms were present.

TABLE V. PRODUCTION OF HYDROGEN SULPHIDE AS DETERMINED BY PEPTONE MEDIA PLUS LEAD ACETATE STRIPS AND BY KLIGLER'S IRON AGAR

Original location of organism	Growth on peptone	H ₂ S production	Growth on Kligler's agar	H ₂ S production
Taber	No growth	0	White vis- cous growth	0
Taber (Lt.)	Turbid growth	+	Viscous growth	+
Vauxhall	No growth	0	Viscous growth	0
Vauxhall (Lt.)	Turbid growth + pellicle	+	Viscous growth	0
Brooks	No growth	0	Viscous growth	0
Youngstown	Turbid growth + pellicle	+	Viscous growth	++
Gleichen	Turbid growth	0	Heavy growth	++
Halkirk	Turbid growth	0	Heavy viscous growth	+
Strome	Turbid growth + pellicle	+	Heavy growth	++
Airdrie	Turbid growth + pellicle	+	Heavy viscous growth	++
Edmonton	Turbid growth	0	Heavy growth	0
Waskatenau	Turbid growth + pellicle	+	Heavy growth	++
Breton	Turbid growth	0	Heavy growth	++
Lac La Biche	No growth	0	Heavy growth	++

TABLE 1. Summary of the results of the analysis of variance for the effect of the treatment on the response of the different groups of subjects.

TABLE 1

1

Treatment		Response		Significance	
Group	Mean	Group	Mean	Group	Mean
1	1.0	1	1.0	1	1.0
2	1.0	2	1.0	2	1.0
3	1.0	3	1.0	3	1.0
4	1.0	4	1.0	4	1.0
5	1.0	5	1.0	5	1.0
6	1.0	6	1.0	6	1.0
7	1.0	7	1.0	7	1.0
8	1.0	8	1.0	8	1.0
9	1.0	9	1.0	9	1.0
10	1.0	10	1.0	10	1.0
11	1.0	11	1.0	11	1.0
12	1.0	12	1.0	12	1.0
13	1.0	13	1.0	13	1.0
14	1.0	14	1.0	14	1.0
15	1.0	15	1.0	15	1.0
16	1.0	16	1.0	16	1.0
17	1.0	17	1.0	17	1.0
18	1.0	18	1.0	18	1.0
19	1.0	19	1.0	19	1.0
20	1.0	20	1.0	20	1.0
21	1.0	21	1.0	21	1.0
22	1.0	22	1.0	22	1.0
23	1.0	23	1.0	23	1.0
24	1.0	24	1.0	24	1.0
25	1.0	25	1.0	25	1.0
26	1.0	26	1.0	26	1.0
27	1.0	27	1.0	27	1.0
28	1.0	28	1.0	28	1.0
29	1.0	29	1.0	29	1.0
30	1.0	30	1.0	30	1.0
31	1.0	31	1.0	31	1.0
32	1.0	32	1.0	32	1.0
33	1.0	33	1.0	33	1.0
34	1.0	34	1.0	34	1.0
35	1.0	35	1.0	35	1.0
36	1.0	36	1.0	36	1.0
37	1.0	37	1.0	37	1.0
38	1.0	38	1.0	38	1.0
39	1.0	39	1.0	39	1.0
40	1.0	40	1.0	40	1.0
41	1.0	41	1.0	41	1.0
42	1.0	42	1.0	42	1.0
43	1.0	43	1.0	43	1.0
44	1.0	44	1.0	44	1.0
45	1.0	45	1.0	45	1.0
46	1.0	46	1.0	46	1.0
47	1.0	47	1.0	47	1.0
48	1.0	48	1.0	48	1.0
49	1.0	49	1.0	49	1.0
50	1.0	50	1.0	50	1.0
51	1.0	51	1.0	51	1.0
52	1.0	52	1.0	52	1.0
53	1.0	53	1.0	53	1.0
54	1.0	54	1.0	54	1.0
55	1.0	55	1.0	55	1.0
56	1.0	56	1.0	56	1.0
57	1.0	57	1.0	57	1.0
58	1.0	58	1.0	58	1.0
59	1.0	59	1.0	59	1.0
60	1.0	60	1.0	60	1.0
61	1.0	61	1.0	61	1.0
62	1.0	62	1.0	62	1.0
63	1.0	63	1.0	63	1.0
64	1.0	64	1.0	64	1.0
65	1.0	65	1.0	65	1.0
66	1.0	66	1.0	66	1.0
67	1.0	67	1.0	67	1.0
68	1.0	68	1.0	68	1.0
69	1.0	69	1.0	69	1.0
70	1.0	70	1.0	70	1.0
71	1.0	71	1.0	71	1.0
72	1.0	72	1.0	72	1.0
73	1.0	73	1.0	73	1.0
74	1.0	74	1.0	74	1.0
75	1.0	75	1.0	75	1.0
76	1.0	76	1.0	76	1.0
77	1.0	77	1.0	77	1.0
78	1.0	78	1.0	78	1.0
79	1.0	79	1.0	79	1.0
80	1.0	80	1.0	80	1.0
81	1.0	81	1.0	81	1.0
82	1.0	82	1.0	82	1.0
83	1.0	83	1.0	83	1.0
84	1.0	84	1.0	84	1.0
85	1.0	85	1.0	85	1.0
86	1.0	86	1.0	86	1.0
87	1.0	87	1.0	87	1.0
88	1.0	88	1.0	88	1.0
89	1.0	89	1.0	89	1.0
90	1.0	90	1.0	90	1.0
91	1.0	91	1.0	91	1.0
92	1.0	92	1.0	92	1.0
93	1.0	93	1.0	93	1.0
94	1.0	94	1.0	94	1.0
95	1.0	95	1.0	95	1.0
96	1.0	96	1.0	96	1.0
97	1.0	97	1.0	97	1.0
98	1.0	98	1.0	98	1.0
99	1.0	99	1.0	99	1.0
100	1.0	100	1.0	100	1.0

Reduction of litmus milk:

Litmus milk was unchanged after 14 days of incubation.

Relation of organism growth to reaction:

The amount of growth appearing on mannitol agar adjusted to a series of pH's is presented in Table VI. Plates 8 and 9 depict the type of growth that was observed with some of the cultures studied. From Table VI it can be seen that growth of the typical Azotobacter cultures stopped somewhere between 5.7 and 6.2. Plate 8 shows the results obtained using Taber and Brooks cultures.

It is of interest to note that growth is fairly uniform across the pH range in which it occurred and stopped decisively when the lower pH limits were reached. Pigmentation seemed to be affected at a pH of 6.2 in the Brooks culture but not in the Taber.

The relation of reaction to the growth of the smaller nitrogen-fixing organisms differs from that of the typical Azotobacter. These organisms can apparently withstand lower reactions than the typical Azotobacter.

Plate 9, Fig. A, shows the Strome culture which grew across the complete pH range of 4.9 to 7.9. Fig. B depicts the growth of the Edmonton culture which was inhibited at a pH of 4.9. These organisms, although growing at a pH below that of the typical Azotobacter, appear to vary somewhat in the amount of acidity which they can withstand.

Optimum temperature of growth and thermal death point:

The data showing effect of temperature upon the development of the nitrogen-fixing organisms under study are presented in Table VII. The optimum growth temperatures for the typical Azotobacter cultures appears

TABLE VI. RELATION OF ORGANISM GROWTH TO REACTION (pH) OF MEDIA GROWTH
ON MANNITOL AGAR MEDIA ADJUSTED TO VARIOUS pH'S

Name of organism	p H o f m e d i a							
	4.9	5.4	5.7	6.2	6.7	7.1	7.5	7.9
Taber	0	0	0	+++	+++++	++++	++++	+++
Taber (Lt.)	0	+	++	++	+++	++	++	+
Vauxhall	0	0	0	+++	++++	+++	++++	+++
Vauxhall (Lt.)	0	+	++	++	++	+++	+++	+++
Brooks	0	0	0	++++	++++	++++	++++	+++
Youngstown	++	++	++	++	+++	+++	++	++
Gleichen	0	+	+	++	++	+	+	0
Halkirk	++	++	++	++	++++	++++	+++	0
Strome	+	++	++	++	++	++	++	++
Airdrie	++	+	+	++	++	++	+	0
Edmonton	0	+	+	++	++	++	++	+
Waskatenau	++	++	++	++	++	++	++	++
Breton	+	+	++	++	++	++	++	++
Lac La Biche	++	++	++	++	+++	+++	+++	+

TABLE VII. THE EFFECT OF TEMPERATURE ON THE GROWTH OF AEROBIC,
NON-SYMBIOTIC NITROGEN-FIXING ORGANISMS

Original location of organism	I n c u b a t i o n t e m p e r a t u r e					
	37°C	28°C	25°C	18°C	8°C	4°C
Taber	++	++++	+++	++	0	0
Taber (Lt.)	++	++	+	++	0	0
Vauxhall	++	++++	+++	++	0	0
Vauxhall (Lt.)	+	++	++	++	0	0
Brooks	++	+++	++	+	0	0
Youngstown	+	++	++	+	0	0
Gleichen	+	++	++	++	+	0
Halkirk	+	++	+++	++	+	0
Strome	+	++	++	+	+	0
Airdrie	+	++	++	+	+	0
Edmonton	+	++	++	++	+	0
Waskatenau	+	++	++	++	+	0
Breton	+	++	++	++	+	0
Lac La Biche	+	++	++	++	0	0

PLATE 8. RELATION OF AZOTOBACTER GROWTH TO REACTION (pH) OF THE MEDIUM

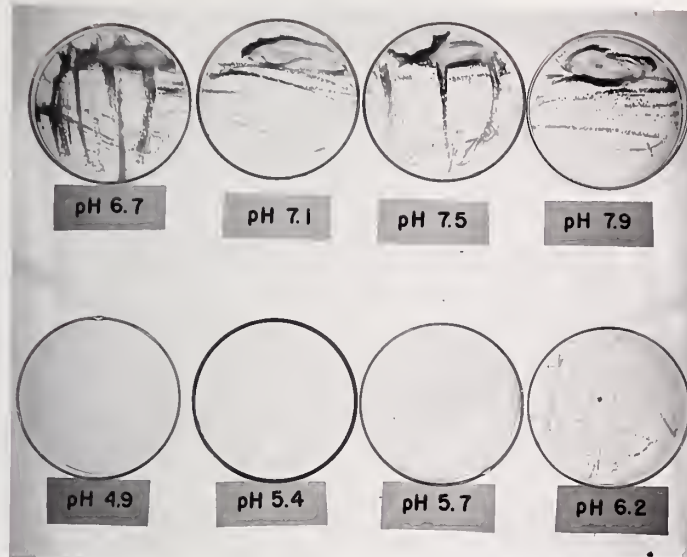


Figure A. Effect of pH on growth of organisms isolated from Taber soil.

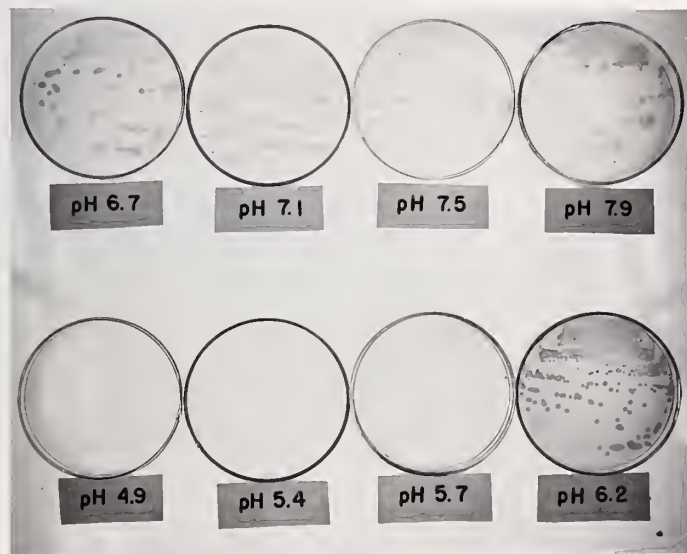


Figure B. Effect of pH on growth of organisms isolated from the Brooks irrigation area.

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PLATE 9. RELATION OF ORGANISMS' GROWTH TO REACTION (pH) OF THE MEDIUM
USING THE SMALLER NITROGEN FIXING ORGANISMS AS INOCULUM

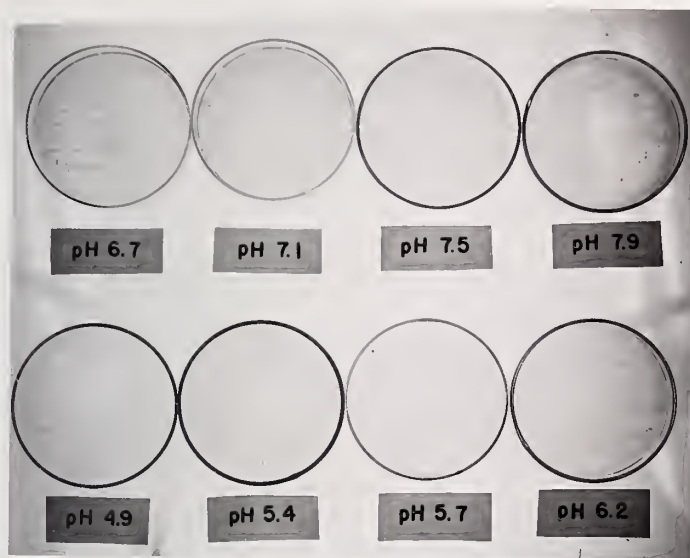


Figure A. Growth of culture isolated from Strome soil.



Figure B. Growth of culture isolated from Edmonton soil.

to be slightly higher than that of the smaller nitrogen-fixing organisms. The smaller organisms also develop at a lower incubation temperature than do the typical Azotobacter. These smaller organisms are capable of a very slow rate of growth at 8°C.

The thermal death point of the nitrogen-fixing organisms was determined. In all cases it was found to lie between 50 and 55°C.

Nitrogen fixation:

A microkjeldahl procedure was used to determine the nitrogen-fixing ability of the organisms. Visible growth occurring on a completely nitrogen-free medium is a fairly safe criterion of the ability of an organism to fix atmospheric nitrogen. Quantitative estimates of the amount of nitrogen fixed by a pure culture of the organism are of little practical value for determining nitrogen fixation under field conditions. In the laboratory, however, nitrogen-fixing determinations of pure cultures of the organisms is useful for corroborating the visual observations concerning the ability to develop in a nitrogen-free medium. It can also be used as a factor in the classification of an organism.

Table VIII presents the data obtained from the microkjeldahl determination of the amount of atmospheric nitrogen fixed by pure cultures of approximately 0.75 mgm. of bacteria inoculated into 5 ml. of a 2 percent mannitol nutrient solution.

The nitrogen fixed in these experiments varied from 1.28 mgm. for the Vauxhall cultures down to 0.03 mgm. for the culture obtained from Halkirk soil. To determine the mgm. nitrogen fixed per gram of mannitol, the amount of nitrogen fixed must be multiplied by 10. The Taber culture fixed 10.6 mgm. nitrogen per gram of mannitol, Vauxhall 12.8, and Brooks 1.65. The values for Vauxhall and Taber coincide with those

TABLE VIII. ATMOSPHERIC NITROGEN FIXED BY 2 ML. OF NON-SYMBIOTIC
NITROGEN-FIXING ORGANISMS AFTER 48 HOURS INCUBATION IN
5 ML. OF 2 PERCENT MANNITOL SOLUTION

Original location of organism	Treatment	Nitrogen content				Average difference
		I	II	III	IV	
Taber	Control	0.13	0.25	0.22	0.14	1.060
	Inoc.	1.19	1.19	1.35	1.25	
Vauxhall	Control	0.17	0.17	0.17	0.18	1.278
	Inoc.	1.54	1.49	1.19	1.58	
Brooks	Control	0.18	0.18	0.17	0.22	.165
	Inoc.	0.36	0.41	0.27	0.37	
Youngstown	Control	0.26	0.19	0.24	0.19	.087
	Inoc.	0.27	0.33	0.34	0.29	
Gleichen	Control	0.08	0.08	0.09	0.10	.390
	Inoc.	0.47	0.47	0.50	0.47	
Halkirk	Control	0.03	0.03	0.03	0.02	.020
	Inoc.	0.05	0.07	0.03	0.04	
Strome	Control	0.12	0.11	0.14	0.16	.105
	Inoc.	0.18	0.32	0.30	0.15	
Airdrie	Control	0.17	0.17	0.15	0.14	.055
	Inoc.	0.24	0.25	0.18	0.18	
Edmonton	Control	0.16	0.25	0.10	0.10	.312
	Inoc.	0.50	0.50	0.43	0.43	
Waskatenau	Control	0.10	0.13	0.12	0.10	.030
	Inoc.	0.17	0.14	0.15	0.11	
Lac La Biche	Control	0.22	0.21	0.21	0.23	.052
	Inoc.	0.27	0.19	0.30	0.32	
Breton	Control	0.13	0.18	0.18	0.18	.060
	Inoc.	0.29	0.27	0.16	0.19	

(continued)

TABLE VIII. (continued)

PART B. ANALYSIS OF VARIANCE

Source of variance	D.F.	S.S.	M.S.	F.
Soils	11	4.5493	0.4181	1.167
Treatments	1	2.1780	2.1780	6.07
Soils + treatments	11	3.9459	0.3587	
Error	72	.2171	.0030	
Total	95	10.9403		

L. S. D. at 5 percent = 0.078.

generally obtained by other workers for typical Azotobacter. The low value for the Brooks culture was unexpected.

The fixation values for the smaller organisms did not exceed 4 mgm. nitrogen per gram of mannitol. The Lac La Biche, Airdrie, Halkirk, and Breton cultures did not fix nitrogen in significant amounts at the 5 percent level of significance.

By the use of this method of inoculation with a fairly large mass of organisms it was possible to reduce the incubation period from the generally recommended 28-day period to one of 48 hours. This results in a saving of research time and also gives a more reliable estimate of the ability of an organism to fix nitrogen. If an organism fixes only small amounts of nitrogen it is not only very difficult to measure any increase accurately with the macrokjeldahl method but during a prolonged incubation period considerable amounts of ammonia could be absorbed from the air. Therefore, even if an increase in the total nitrogen content of the medium is recorded, the source of this nitrogen may be physical absorption of ammonia rather than biological fixation. The use of a 48-hour incubation period and a smaller volume of liquid virtually eliminates this source of error.

The organisms isolated from Halkirk soil fixed a very small quantity of nitrogen. The original nitrogen content of the inoculum was very low, and one could probably expect little fixation. The inoculum, consisting of a washed resting cell suspension of organisms, was standardized to 15 percent transmittance in a colorimeter. During the washing and centrifugation of these organisms, copious amounts of slime were observed. This slimy growth, although affecting the transmittance of light, probably did not contain as many bacterial cells as slime-free growth. This could account for the original and final low nitrogen content in this case.

A possible source of error in all of the determinations was the probability of unequal distribution of the cells in the original inoculum. On centrifugation the cells packed considerably. They were resuspended in the liquid by agitation with a sterilized wire loop. If all the clots were not broken down, the possibility of incorrect sampling upon pipetting existed. To reduce this source of error the method should be adapted to use with a sterilized mechanical stirrer which would ensure a uniform distribution of cells.

GENERAL DISCUSSION

A summary of the characteristics of the organisms will facilitate their classification. Cultures isolated from Taber cultivated-irrigated and cultivated non-irrigated, and from Brooks and Vauxhall irrigated soils grew on a mannitol agar culture medium, producing large, mucous, coalescent colonies. The colonies had a greyish-brown pigment insoluble in water. On sodium benzoate agar, 3 - 4 mm. flat circular colonies, producing a black soluble pigment, were formed. The growth on mannitol agar slants was brown filiform showing some evidence of slime production. On sodium benzoate slants filiform to beaded growth produced a soluble black pigment.

Growth was very limited in nutrient broth, peptone water, and on nutrient agar.

The Gram-negative 2 - 3 u. yeastlike rods and the plump 2 - 4 u. rods were found to predominate. They occasionally existed as 4 - 6 u. giant cells or as long rods up to 7 u. in length. Refractive granules were present and the organisms were definitely motile.

Potassium nitrate inhibited rather than enhanced growth on mannitol agar. Starch was utilized. Gelatin was not liquified; nor was indol produced from tryptophane. Hydrogen sulphide was not produced in any of the typical Azotobacter cultures tested. Litmus milk remained unreduced after 14 days.

Growth stopped very decisively at a pH between 5.7 and 6.2. It was not completely inhibited at a pH of 7.9. Growth occurred at a pH of approximately 7.0 at an optimum rate.

The organisms developed more profusely at 28°C. than at either 25°C. or 37°C. Growth did not occur at 8°C. The thermal death temperature for a ten-minute exposure period was 55°C.

The Taber culture fixed approximately 11 mgm. nitrogen per gram of mannitol, Vauxhall 13, and Brooks less than 2 mgm. The Brooks culture tended to grow slightly more watery and produced less pigment after prolonged growth on synthetic media. These factors and the lower rate of nitrogen fixation were the only ones in which the Brooks culture differed from Taber and Vauxhall. The Brooks cultures resembled very closely the Vauxhall and Taber cultures on initial isolation. Therefore, all of these larger nitrogen-fixing organisms probably belong to one species. A comparison of the results obtained in this study with either Hofer's (39) or Jensen's (45) classification established these aerobic non-symbiotic nitrogen-fixing organisms as Az. chroococcum.

The classification of the smaller nitrogen-fixing organisms isolated from Alberta soils presents more difficulties. The dust plate technique is an inadequate test for the occurrence of this organism. The minute colonies, if they do occur, are obscured by the soil particles. Mannitol agar plates inoculated with a mannitol water solution, in which

the organisms had been growing, developed small, unpigmented, flat, circular, mucilaginous colonies. Colony growth on sodium benzoate plates was similar to that on mannitol agar except in the Breton cultivated and the Lac La Biche cultivated and virgin soils. Cultures from these soils produced a dark brown soluble pigment.

The organisms did not stain well with safranin, basic fuchsin, or methylene blue. These stains did not penetrate the cells. This resulted in a halo effect with the cells staining only at the edges.

The organisms were Gram-negative in all cases. Stained with carbol fuchsin the cells were found to be 0.75 - 1 u. by 1.5 - 2 u. coccoid rods which sometimes formed diploids. Some variation occurred. The Strome culture which was isolated as a small rod was at times observed in a rounded shape which was almost yeastlike in appearance.

Youngstown, Lac La Biche, and Taber (Lt.) were found to be slightly motile. None of the other organisms were motile under the conditions of the hanging drop motility test.

The addition of a small quantity of nitrate enhanced growth. Starch was readily hydrolysed by these organisms. Gelatin was not liquified; indol was not produced; nor was litmus milk reduced. Hydrogen sulphide was produced by all of the organisms except Edmonton.

The organisms grew at a more acid reaction than the Azotobacter chroococcum isolated. Growth occurred down to a pH of 4.9. The optimum temperature for growth was slightly lower than that of the typical Azotobacter. Growth was more profuse at 25°C. than at 28°C. and still occurred at a temperature of 8°C. Acid was not produced from sugars and alcohols.

The differences in nitrogen fixation of organisms has to be

attributed to either species or strain variations or to such biological variable as the activity of the organisms in the inoculum at the time of inoculation. Nitrogen fixation by the non-symbiotic nitrogen fixers appears to be growth bound. Therefore it should be possible to correlate visible growth with nitrogen fixation. No differences in visible growth were noted between plate cultures of organisms such as those of Strome and Airdrie soils. However, in the nitrogen-fixing determination the Strome culture proved to be an active fixer, whereas Airdrie, although it apparently fixed some nitrogen, did not fix amounts significant at the 5 percent level.

Comparison of the morphological and biochemical characteristics of these smaller nitrogen-fixing organisms shows that although there is some variation it tends to be spasmodic and can probably be attributed to improper methods, adaption of the organisms, or some strain differences. The differences between the organisms are not enough to merit species differentiation. Therefore it can be concluded that all the smaller nitrogen-fixing organisms isolated from Alberta soils belong to one species. The smaller size could be reconciled with some of the cell types as described by Jensen (45). The development at a pH below 6.0 differs from other results obtained with Azotobacter in America. However, Jensen describes the strain of Az. beijerinckii which develop at a pH of 4.5.

The Az. chroococcum isolated from Alberta soils did not produce hydrogen sulphide. The smaller nitrogen-fixing organisms did produce hydrogen sulphide. If they do belong to the Azotobacter, the only species with which they could be reconciled is Az. beijerinckii, which is described as being non-motile. The organisms under study were found to be motile in at least three cases. This casts some doubt on the name Az. beijerinckii for these organisms.

The soils of Alberta studied in this investigation contained aerobic non-symbiotic nitrogen-fixing organisms. Az. chroococcum were found to be present only in the brown soil zone. A smaller non-pigment producing organism, isolated from all Alberta soils investigated, and capable of developing on nitrogen-free media could tentatively be designated as Az. beijerinckii.

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APPENDIX

Microkjeldahl Determination of Nitrogen

The microkjeldahl methods of Galston (25) and Milton (60) using selenium and mercury catalysts in a weak digestion solution, were tested but found to be unsatisfactory due to the limited range over which nitrogen could be determined.

The procedure, finally adopted, employed the principles of a method developed by Polly (63). The nitrogen-containing material was digested with 1.5 ml. concentrated sulphuric acid, .5 ml. of 10 percent mercuric sulphate, and 1.5 gm. potassium sulphate. The digestate was made up to volume in a Folin digestion tube. After amalgamating the mercury with 0.2 gm. of zinc, an aliquot of the digestate was diluted and Nesslerized. By varying the proportions of the digestate and distilled water, it was possible to determine the total nitrogen over a fairly wide range.

Reagents Employed

Nitrogen standard two mgm. per ml.:

9.4332 gm. of oven-dried $(\text{NH}_4)_2\text{SO}_4$ made up to 1 litre with nitrogen-free water.

Nitrogen-free water:

Distilled water which was run through a cation exchange column, was stored in a 20 litre pyrex flask equipped with a 5 N. H_2SO_4 trap to prevent the entrance of NH_3 .

Concentrated H_2SO_4 , Reagent grade.

Potassium sulphate, Reagent grade.

10 percent HgSO_4 :

12 ml. of concentrated H_2SO_4 were diluted to 100 ml. with water and 10 gm. of HgO were dissolved in this solution.

Sodium hydroxide 5.0 N., Reagent grade.

Nessler's solution:

Folin-Wu commercial concentrated solution diluted 1 to 4 with 10 percent NaOH .

Hydrogen peroxide:

30 percent nitrogen-free superoxol.

Sodium sulphide 4 percent:

40 gm. Na_2S per litre of H_2O .

Digestion Procedure

Digestion was carried out in 100 ml. pyrex volumetric flasks. The use of volumetrics eliminated the need for transferring the digestate from the digestion flasks.

Samples ranging in nitrogen content from 0.1 mg. per ml. to 12.0 mg. per ml. were pipetted into volumetric flasks. Then 0.5 gm. K_2SO_4 , 0.5 ml. of HgSO_4 solution, and 1.5 ml. of concentrated H_2SO_4 were added. After digesting for 30 minutes and cooling, 5 drops superoxol were added and digestion continued for another 10 minutes. The flasks were allowed to cool, the sides were washed down with 5 ml. of H_2O and the mercury was precipitated.

Attempts to use Polly's method of amalgamating the mercury with zinc proved futile. The effervescence caused by the reaction

between the zinc and the sulphuric acid, releasing hydrogen, continued even after 10 minutes of boiling at which time the sulphuric acid was consumed and the digestate ruined. Varying the zinc concentration did not prove satisfactory so other methods of precipitating the mercury were investigated. The use of sodium thiosulphate occasionally gave a turbid suspension upon Nesslerization. Sodium sulphide was then employed using 4 ml. of a 4 percent solution of sodium sulphide. This proved the most satisfactory, in that it caused formation of a flocculent precipitate of mercuric sulphide which settled rapidly and completely.

The sodium sulphide was added rapidly to the swirling solution by the use of an automatic pipette ensuring thorough mixing. The solution was then boiled for two minutes to expel the hydrogen sulphide formed. After cooling, the sides of the volumetric were once more washed down with water. Then 7.5 ml. of 5 N. NaOH were added to the swirling solution and well mixed. This still left the digestate with a slightly acidic reaction preventing ammonia losses. The flasks were made up to volume and allowed to stand till the precipitate settled. This required approximately twenty minutes. The precipitate can also be removed by centrifuging a portion of the digestate for three minutes at approximately 2,500 r.p.m.

Color Development -- Nesslerization

The concentration of nitrogen that can be accurately determined by Nesslerization is limited by the fact that the percent transmittance reading of the Nesslerized color must lie within the maximum efficiency range of the colorimeter employed -- in this case a Bausch and Lomb Spectronic 20.

In order to do this three different sets of dilutions were

employed resulting in colorimetric curves designated as the micro, the general and the macro.

For the general curve, 10 ml. of the digestate were pipetted into 50 ml. volumetric flasks. Thirty ml. of nitrogen-free water was then added to the volumetrics. At one-minute intervals, 5 ml. of Nessler's solution were added from an automatic pipetted and the solution in the flasks immediately made up to volume. The percent transmittance was read on the colorimeter at 480 mμ., Hawk et al. (36). A blank, containing all the reagents except nitrogen was used to set the colorimeter reagents except nitrogen, was used to set the colorimeter at 100 percent transmittance before each reading.

For the microcurve 20 cc. of digestate were pipetted into 25 ml. volumetrics, and Nesslerized with 3.5 ml. of Nessler's solution. The macrocurve consisted of 5.0 ml. of digestate diluted with 80 ml. water and Nesslerized with 10 ml. Nessler's reagent and immediately made up to volume with water. The proportions of Nessler's reagent to digestate for the various concentrations was calculated to give the required basicity as previously mentioned and was also determined by the trial and error method.

Testing the Microkjeldahl Determination of Nitrogen for Its Applicability to Nitrogen Fixation Experiments

The agreement of the replications was tested using one ml. of the two mg. per ml. standard. In ten replicates the reading varied from 42 to ± 1 percent transmittance. This consistency of results, using only one concentration, could be fairly easily obtained.

Table IX shows the results of determinations conducted in order to draw the final standard curve on 1 cycle x 70 semilogarithmic paper. The values obtained were plotted for the various dilutions, and all fell on the same curve as seen in the accompanying graph.

The nitrogen values obtained using the described micromethod for determining the nitrogen content of purified casein were used for comparison with values obtained by the Kjeldahl macromethod using the A.O.A.C. (6) technique. The results of the comparison are shown in Table X.

Experience showed that variation in the percent transmittance of 1 percent gave a variation of close to 3 percent in the nitrogen recovered. Attempts will have to be made to reduce this variation by using more replicates and by very careful standardization of the colorimeter using the blank before each reading plus exact matching of the colorimetric tubes.

The method was then tested for its suitability for determining the nitrogen content of bacterial suspension. The organisms in question were grown on a nitrogen-free mannitol medium, washed with distilled water by centrifugation, and then standardized on the colorimeter. A maximum transmittance curve indicated that 585 mu. was the desired wave length. The suspension containing bacteria was adjusted to a concentration which gave 15 percent transmittance at 585 mu., using distilled water as a blank. In the testing of the organism's ability to fix nitrogen, the checks after inoculation had the digesting mixture of sulphuric acid and 10 percent HgSO_4 immediately added to the culture whereas the remainder were incubated at 25°C. for 48 hours in an incubating chamber equipped with a reciprocating shaker.

1. The first part of the report is devoted to a general

description of the situation in the country at the beginning of the year.

2. The second part of the report is devoted to a detailed

analysis of the economic situation in the country.

3. The third part of the report is devoted to a detailed

analysis of the social situation in the country.

4. The fourth part of the report is devoted to a detailed

analysis of the political situation in the country.

5. The fifth part of the report is devoted to a detailed

analysis of the cultural situation in the country.

6. The sixth part of the report is devoted to a detailed

analysis of the scientific situation in the country.

7. The seventh part of the report is devoted to a detailed

analysis of the sports situation in the country.

8. The eighth part of the report is devoted to a detailed

analysis of the health situation in the country.

9. The ninth part of the report is devoted to a detailed

analysis of the environmental situation in the country.

10. The tenth part of the report is devoted to a detailed

analysis of the international situation in the country.

11. The eleventh part of the report is devoted to a detailed

analysis of the future prospects of the country.

12. The twelfth part of the report is devoted to a detailed

analysis of the conclusion of the report.

13. The thirteenth part of the report is devoted to a detailed

analysis of the appendixes of the report.

14. The fourteenth part of the report is devoted to a detailed

analysis of the bibliography of the report.

TABLE IX. DETERMINATION OF THE STANDARD CURVE FOR THE NESSLERIZATION
OF THE KJEIDAHN DIGESTATE

Milligrams N in sample	Dilution	Micrograms N in final Nesslerized solution	% T
8	1/2000	4	42
7	"	3.5	46.5
6	"	3.0	53
5	"	2.5	58
4	"	2.0	67
3	"	1.5	74.5
3.5	1/500	7	23
3.0	"	6	26.5
2.8	"	5.6	30
2.6	"	5.2	--
2.5	"	5.0	33
2.4	"	4.8	--
2.0	"	4.0	42
1.8	"	3.6	46
1.6	"	3.2	51
1.5	"	3.0	52.5
1.4	"	2.8	55
1.2	"	2.4	59
1.0	"	2.0	68
.5	"	1.0	81
.2	"	.4	91
.1	"	.2	96
.05	"	.1	98
.02	"	.04	99
.8	1/125	6.4	24
.6	"	4.8	34
.4	"	3.2	51
.2	"	1.6	72
.1	"	.8	86

FIGURE 2. MICROGRAMS OF NITROGEN AS DETERMINED BY NESSLERIZATION

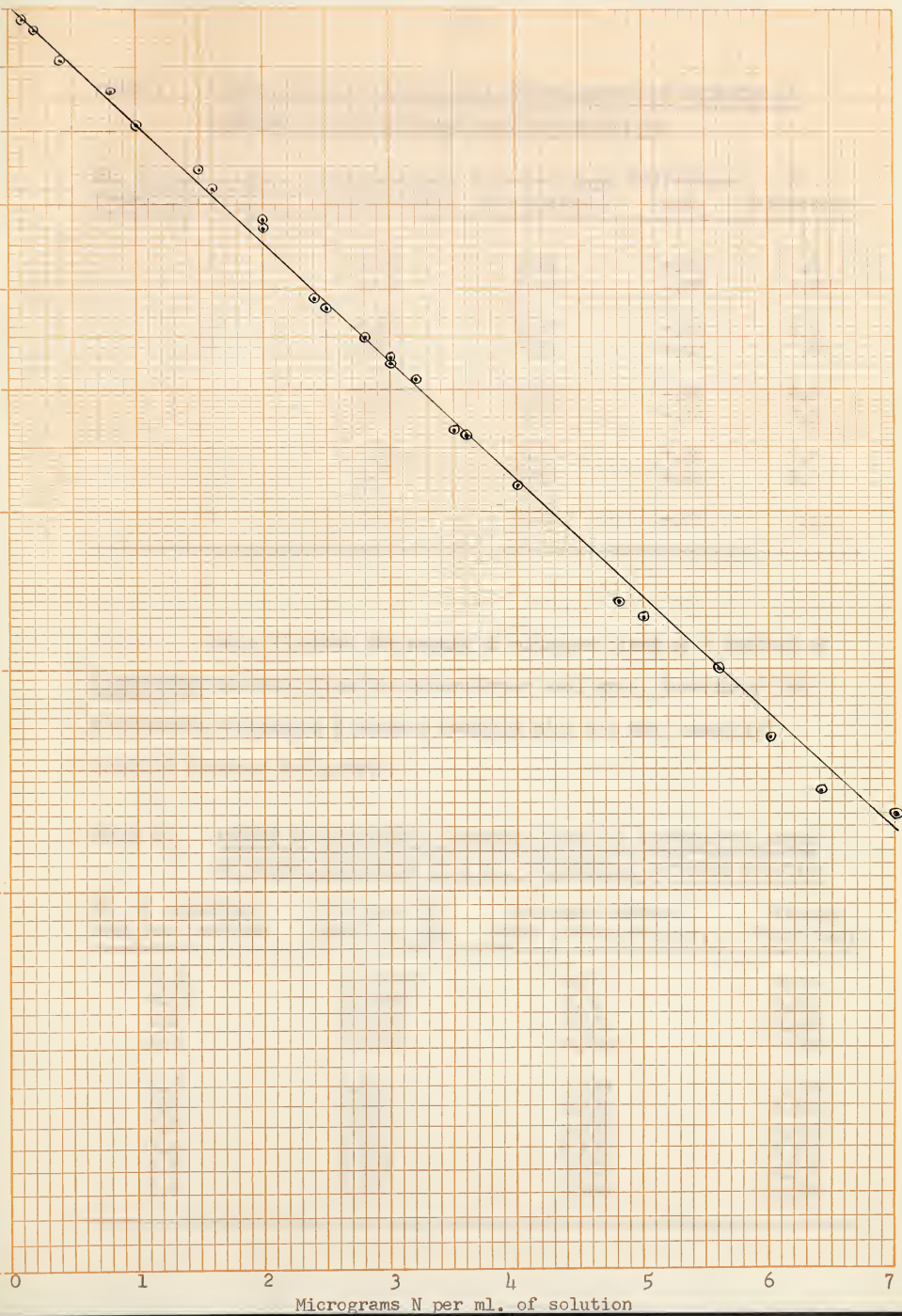




TABLE X. COMPARISON OF MICROKJELDAHL DETERMINATION OF NITROGEN AS
COMPARED TO THE MACROKJELDAHL DETERMINATION

ML. of casein soln. containing 15.15% N	N content mgm. macrokjeldahl	N content mgm. microkjeldahl	Difference mgm.	% Difference
1	1.14	1.15	+.01	.8
1	1.14	1.12	-.02	1.8
2	2.29	2.40	+.11	4.6
2	2.29	2.31	+.02	.87
3	3.40	3.32	-.08	2.3
3	3.40	3.28	-.12	3.5
4	4.54	4.30	+.24	5.2
4	4.54	4.40	+.14	3.0
5	5.67	5.68	+.01	.17

Table XI shows the amount of nitrogen fixed by a species of Azotobacter isolated from the Brooks brown soil area, inoculated into a substrate containing 2 percent mannitol plus all the essential minerals required for growth.

TABLE XI. AMOUNT OF ATMOSPHERIC NITROGEN FIXED BY AZOTOBACTER AFTER
48 HOURS INCUBATION IN 5 ML. OF MANNITOL NUTRIENT SOLUTION

ML. of organisms used for inoculum	Nitrogen in inoculum, mgm.	Nitrogen content after incubation mgm.	Nitrogen fixed mgm.
0.01	0.00091	0.00	0.00
0.01	0.00091	0.05	0.05
0.1	0.0091	0.112	0.103
0.1	0.0091	0.106	0.095
0.5	0.046	0.145	0.099
0.5	0.046	0.175	0.129
1.0	0.091	0.234	0.143
1.0	0.091	0.234	0.143
2.0	0.182	0.361	0.179
2.0	0.182	0.264	0.087

From the table it can be seen that by this method it is possible not only to determine the original nitrogen in quantities of bacteria but also to determine the amount of nitrogen fixed under controlled conditions.

From the foregoing results it can be seen that by the utilization of the described microkjeldahl method for the determination of organic nitrogen, quantitative results can be obtained. When applied to bacteria it provides a convenient means of determining not only the original nitrogen content of a bacterial suspension, but also the nitrogen-fixing ability of the non-symbiotic nitrogen fixers. The method is flexible enough to allow for changes in: the concentration and pH of the medium employed; the number of bacterial cells used; and the conditions of incubation such as duration and temperature of incubation. This allows one to perform a comprehensive study of not only the relative nitrogen-fixing ability of the organisms but also facilitates the study of the kinetics of the fixation as described above.

The method is not limited to bacterial cells but can profitably be employed wherever the nitrogen content of organic substances having a fairly wide range of nitrogen must be determined by using a Nesslerization procedure.

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